ISOLATION AND CHARACTERIZATION OF A GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN PLASMIN

By

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This dissertation is dedicated
to the memory of my father
Thomas J. Broder

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KEY TO ABBREVIATIONS

α_2-AP/α_2-PI	alpha-2-antiplasmin
ATCC	American typed culture collection
BSA	bovine serum albumin
cpm	counts per minute
DNase	deoxyribonuclease
EACA	epsilon aminocaproic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked-immunosorbant-assay
FPLC	fast performance liquid chromatography
g	gravity
Glu-plasminogen	native human plasminogen with NH_2-terminal glutamic acid
HC	heavy chain of plasmin
HRGP	histidine-rich glycoprotein
IgG	immunoglobulin class G
KD	kilodalton
K_D	dissociation constant
KIU	kallikrein inhibitor unit
LBS	lysine-binding site
LC	light chain of plasmin
Lys-plasminogen	proteolytically modified form of Glu-plasminogen with NH_2-terminal lysine
M	molar

Mini-PLA	low molecular weight plasmin
Mini-PLG	low molecular weight plasminogen
mM	millimolar
μM	micromolar
μm	micrometer
MOPS	(3-[N-Morpholino]propanesulfonic acid)
M_r	relative molecular weight
NIH u	National Institute of Health unit
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PLA	plasmin
PLG	plasminogen
p	pico
PMSF	phenylmethylsulfonylfloride
pNpGB	p-nitrophenyl p-guanidinobenzoate HCl
PPACK	Phe-Pro-Arg-chloromethylketone
RNase	ribonuclease
S-2251	H-D-Val-Leu-Lys-paranitroanilide
SDS	sodium dodecylsulfate
SK	streptokinase
tPA	tissue-type plasminogen activator
Tris	(Tris[hydroxymethyl]aminomethane
UK	urokinase
VBS-gel	Veronal-buffered saline plus gelatine
VPLCK	D-Val-Phe-Lys-chloromethyl ketone

Abstract of Dissertation Presented to the Graduate School
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The expression of a specific receptor for the key human fibrinolytic enzyme plasmin on the surface of the group A streptococcal strain 64/14 is reported. The receptor was specific for plasmin, and demonstrated no significant reactivity with the zymogen form of the molecule (Glu-plasminogen). Bacterial bound plasmin retained its enzymatic activity, and could not be inhibited by the physiological regulator (α_2-antiplasmin). The receptor demonstrated a high affinity for plasmin (K_D=1.0x10^{-10} M), and binding was maximal at physiologic pH and ionic strength. Furthermore, the receptor-ligand complex was reversibly inhibitable by ϵ-aminocaproic acid and L-lysine. The binding of plasmin to this group A streptococcus was found to occur primarily through interactions with the heavy chain of the plasmin molecule, and was dependent on a specific conformation of the ligand. A functionally active plasmin receptor was obtained from strain 64/14 bacteria by an enzymatic extraction with mutanolysin. Plasmin binding activity was expressed predominantly in a protein having an M_r of approximately 41,000

daltons. The plasmin receptor demonstrated no plasminogen activator activity. A functionally active plasmin receptor protein was purified by affinity chromatography using immobilized plasmin and specific elution with L-lysine or EACA. The strain 64/14 plasmin receptor was compared with secreted streptokinase proteins from five streptococcal isolates including strain 64/14. Only the plasmin receptor-plasmin complex was found to be sensitive to L-lysine or EACA. Polyclonal rabbit and mouse anti-plasmin receptor antibodies were prepared, as well as polyclonal anti-group C streptokinase antibodies. Using these antibodies as well as a bank of mouse monoclonal anti-group C streptokinase antibodies, the 41,000 dalton plasmin receptor protein from strain 64/14 was shown to be antigenically unrelated to either group A or C streptokinase. Thus the strain 64/14 streptococcal receptor for human plasmin is physicochemically, functionally, and antigenically distinct from streptokinase. The importance of a specific receptor for human plasmin on pathogenic streptococci is unclear; however, it may provide a mechanism for the capture of the potent enzyme plasmin which may confer additional invasive properties to the bacteria.

CHAPTER ONE
INTRODUCTION

Introduction

Plasmin, a serine protease, is the key component of the mammalian fibrinolytic enzyme system. The main physiological role of the fibrinolytic system is the dissolution of fibrin clots formed in blood vessels. Milstone, in 1941, determined that the lysis of fibrin, by the streptococcal substance described by Tillett and Garner in 1933, was dependent on a 'lytic factor' in human serum. This was later followed by the discovery that the lytic factor was an enzyme precursor, in human plasma, that was converted to an active enzyme by a component in the streptococcal fluid (Christensen, 1945; Kaplan, 1944). This precursor was called plasminogen, the enzyme plasmin, and the streptococcal factor streptokinase (Christensen and Macleod, 1945). The zymogen precursor plasminogen, molecular weight approximately 92,000 daltons, is a single-chain glycosylated protein containing 790 amino acids in known sequence and containing 24 disulfide bridges (Brogden et al., 1973; Sottrup-Jensen et al., 1978; Wiman, 1973, 1977).

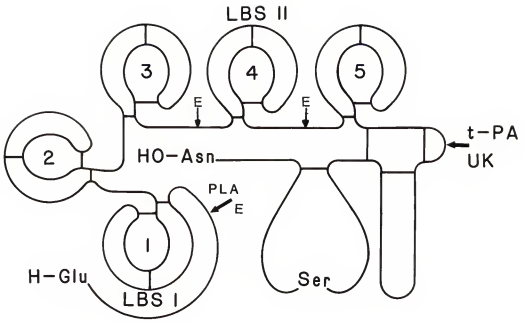
Plasmin(ogen) Structure

Native plasminogen (Glu-plasminogen) has glutamic acid as the NH_2-terminal residue, but is readily converted by the action of plasmin to modified forms of plasminogen which are commonly called Lys-plasminogen.

These modified forms of plasminogen have lysine, valine, or methionine as their N-terminal amino acid (Wallén and Wiman, 1970, 1972). These modifications occur by the hydrolysis of the Arg_{67}-Met_{68}, Lys_{76}-Lys_{77}, or Lys_{77}-Val_{78} peptide bonds. The generation of plasmin from plasminogen occurs through the specific cleavage of a single Arg-Val bond corresponding to the Arg_{560}-Val_{561} positions (Robbins et al., 1967). This cleavage generates a two chain active plasmin molecule consisting of a heavy chain and light chain held together by disulfide linkages (Groskopf et al., 1969; Wiman, 1973) (see Figure 1-1). The light chain of plasmin has a molecular weight of approximately 25,000 daltons (Robbins and Summaria, 1970; Wiman, 1977) and contains the serine active site. The heavy chain of plasmin has a molecular weight of approximately 63,000 daltons (Robbins and Summaria, 1970), and amino acid sequencing revealed a structure containing 5 homologous triple loop structures known as kringles (Sottrup-Jensen et al., 1978).

Several specific compounds such as lysine, ϵ-aminocaproic acid (EACA), trans-4-aminomethycyclohexanecarboxylic acid (transexamic acid), and C-terminal lysine peptides bind to certain sites on the plasmin(ogen) molecule. These specific sites are the characteristic 'lysine-binding sites' distinct from the catalytic site (Thorsen, 1975). These compounds affect the properties of plasminogen and plasmin, and play an important role in determining this zymogen-enzyme system's physiological specificity. Chapter Three will go into more detail on the nature of plasmin(ogen)'s lysine binding sites. Affinity chromatography of defined fragments of plasminogen on lysine Sepharose has demonstrated that these 'lysine-binding sites' are located in the portion of the plasmin molecule which becomes the heavy chain upon activation. The

Figure 1-1. Schematic representation of the human Glu-plasminogen molecule. H-Glu: N-terminal glutamic acid residue; HO-Asn: C-terminal asparagine residue; Ser: serine residue of the enzyme active site; t-PA and UK: plasminogen activation cleavage site on Glu-plasminogen; LBS-I and LBS-II: lysine-binding sites; 1-5: kringle domains numbers 1 through 5. E: elastase cleavage sites. Adapted from Collen (1980).


LBS II
3
4
5
E
E
t-PA
UK
2
HO-Asn
PLA
E
1
H-Glu
LBS I
Ser

native Glu-plasminogen molecule contains one high affinity lysine-binding site (K_d = 9 μM) and five weaker lysine-binding sites (K_d = 5 mM) (Markus et al., 1978a, 1978b). Lys-plasminogen contains one high affinity, one intermediate affinity, and four lower affinity lysine binding sites. The exact number of sites on the plasmin molecule has not been reported. Two of these lysine-binding sites have been mapped to specific regions in the plasminogen molecule. Studies involving equilibrium dialysis experiments on the binding of EACA to isolated fragments of the plasmin(ogen) molecule (see Chapter Three for a complete discussion of these fragments) revealed that the high affinity lysine-binding site was located in the kringle 1 structure, and kringle 4 contained one of the lower affinity sites (Lerch et al., 1980).

Plasminogen Activation

The generation of plasmin from plasminogen is accomplished by plasminogen activators. Three plasminogen activators have been extensively studied. Urokinase (UK) and tissue plasminogen activator (tPA) are proteolytic enzymes (for review see Astrup, 1978); the third, streptokinase (SK), possesses no inherent proteolytic activity. Tissue plasminogen activator, by virtue of its serine protease activity can directly activate plasminogen. This enzyme is present in various tissues and can also bind to fibrin. Urokinase, also a serine protease, is a glycoprotein which has no fibrin binding capacity. Urokinase can also activate plasminogen directly cleaving the Arg_{560}-Val_{561} peptide bond.

Streptokinase is a unique plasminogen activator which is produced by certain streptococci. The only apparent function of streptokinase, since its initial description by Tillett and Garner (1933), is its ability to

activate plasminogen. Unlike the other plasminogen activators, streptokinase has no enzymatic activity. The activation mechanism lies in its ability to form a specific 1:1 stoichiometric complex with plasminogen, as well as with plasmin, which leads to the generation of an active enzyme moiety, presumably through conformational changes in the plasminogen molecule without the cleavage event at the Arg_{560}-Val_{561} peptide bond (Markus et al., 1976), that can in turn act as a plasminogen activator for plasminogen molecules. This is a function neither of the two proteins possesses alone.

Plasmin Regulation

Once generated, plasmin's activity is also carefully regulated under normal physiological conditions. This regulation is accomplished by a specific inhibitor of plasmin known as α_2-antiplasmin (α_2-AP) (Aoki et al., 1977; Collen, 1976; Collen et al., 1975). $Alpha_2$-antiplasmin is a single chain glycoprotein with a molecular weight of approximately 70,000 daltons. α_2-antiplasmin forms a 1:1 stoichiometric complex very rapidly (estimated rate constant of $k_1=3x10^7 M^{-1}s^{-1}$) (Wiman and Collen, 1978) and neutralizes plasmin's activity through a covalent association with the serine residue in the active site of plasmin. A physiological role of α_2-AP as an inhibitor of other proteases other than plasmin appears negligible (Edy and Collen, 1979; Ohlsson and Collen, 1977).

Workers pioneering the techniques of tissue culturing noted that explants of cancer tissue consistently caused proteolytic degradation, liquefying the plasma clots on which they were grown (Carrel and Burrows, 1911; Lambert and Hanes, 1911). Since those early studies the plasminogen-plasmin system, in addition to its role in fibrinolysis, has

been implicated in a variety of normal and abnormal processes which involve the destruction or alteration of the extracellular environment, such as tumor cell growth and invasiveness (for review of this extensive literature see Dano et al., 1985), tissue remodeling, embryogenesis (Beers et al., 1975), ovulation (Strickland and Beers, 1976), and trophoblast implantation (Strickland et al., 1976). In fact, plasmin exhibits broad substrate specificity and in addition to fibrin can hydrolyze components of connective tissue and basement membranes such as laminin, proteoglycans, fibronectin, thrombospondin, and type-V collagen, as well as proteolytically activating other proteases (for review see Knudsen et al., 1986) and several plasma proteins (Marder et al., 1982).

Summary and Specific Aims

In the process of examining human serum from patients for antibody reactivity directed against the streptococcal plasminogen activator streptokinase, from patients who received thrombolytic therapy by streptokinase administration, an interesting observation was made by Dr. M.D.P. Boyle and Dr. R. Lottenberg. In experiments which involved immune precipitations using heat killed, Fc-receptor expressing, group C streptococci it was found that control tests involving incubations of the radiolabeled tracer (^{125}I-plasmin) and bacteria revealed an association of plasmin to bacteria in the absence of any added antibody. Testing other groups of streptococci showed that the group A streptococci displayed the highest level of plasmin binding activity, while demonstrating little binding activity for preparations of ^{125}I-plasminogen.

The ability of certain group A streptococci to produce a plasminogen activator (e.g. streptokinase) and also to express a receptor for the activation product plasmin, may contribute to the invasive properties of these bacteria. This study has been designed to characterize this plasmin receptor phenomenon more completely in order to increase understanding of any potential role in bacterial pathogenesis.

The specific aims of the study are to

1. Identify and characterize a group A streptococcal receptor for human plasmin (Chapter Two).
2. Map the binding site on human plasmin recognized by the bacterial plasmin receptor (Chapter Three).
3. Isolate and purify a functionally active group A streptococcal plasmin receptor (Chapter Four).
4. Compare the group A streptococcal receptor for human plasmin with streptokinase, (Chapter Five), with respect to
    a. Plasminogen activator activity.
    b. Plasmin(ogen) binding domain specificity.
    c. Antigenic relatedness.

CHAPTER TWO
IDENTIFICATION AND CHARACTERIZATION OF A GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN PLASMIN

Introduction

Many group A streptococcal infections are characterized by tissue invasion. A variety of characteristics of these microorganisms contribute to their ability to break down natural tissue barriers and to avoid elimination by the host immune response. Certain surface proteins or secreted products associated with streptococci have been identified that enable these organisms to elude the immune system (Sparling, 1983), and proteins and toxins produced by these bacteria are known to contribute to tissue damage (Ginsburg, 1972; Johnston, 1984; Sparling, 1983). In addition a variety of receptors for host proteins have been described on streptococci. These include receptors for components of the immune system such as Clq (Yarnall et al., 1986), IgG (Kronvall, 1973), and IgA (Russell-Jones et al., 1984), the serum protein fibrinogen (Kronvall et al., 1979), and the stromal structural proteins laminin (Switalski et al., 1984) and fibronectin (Myhre and Krusela, 1983). In this chapter I describe the presence of a specific receptor for human plasmin on certain group A streptococci.

Materials and Methods

Human Plasminogen

Human plasminogen was prepared from human plasma by chromatography on lysine Sepharose and molecular sieving chromatography on Sephadex G-

100 (Lottenberg et al., 1985). Plasminogen was quantified by measuring absorbance using a A_{1cm}^{280nm} value of 17.0 (Nilsson et al., 1982).

Enzymes, Inhibitors and other Reagents

The enzymes urokinase and trypsin were obtained from the Sigma Chemical Company, St. Louis, Mo.; Aprotinin was obtained as Trasylol from Mobay Pharmaceuticals, New York, New York. Phe-pro-arg chloromethylketone (PPACK) was obtained from Cal-Biochem (San Diego, Ca.) P-nitrophenyl, p-guanidobenzoate HCl (pNpGB) was obtained from Sigma Chemical Co, St. Louis, Mo.; human α_2-antiplasmin (α_2-AP) was obtained from American Diagnostica Inc., Greenwich, Connecticut. H-D-Val-leu-lys-paranitroanilide (S-2251) was obtained from Helena Chemical Co., Beaumont, Texas.

Radioiodination of Proteins

Human plasminogen, urokinase, and trypsin were iodinated by a mild lactoperoxidase method using Enzymobeads (Bio-rad Laboratories Richmond, Calif.) as described by Reis et al., (1983). The labeled proteins were separated from free iodine by passage over a G25 column (PD-10 Pharmacia) and collected in 0.15 M Veronal buffered saline pH 7.35 containing 0.001 M Mg^{++}, 0.00015 M Ca^{++} and 0.1% gelatin (VBS-gel). The labeled proteins were stored in aliquots containing 0.02% sodium azide at -20°C. Labeled aliquots were used once and discarded.

Generation of Plasmin

Plasmin was generated from either radiolabeled or unlabeled plasminogen by reaction with urokinase. Three μl of urokinase (Sigma 20 u/ml) was added to a 400 μl solution of 1.0 μM plasminogen containing 0.04 M lysine. The mixture was incubated at 37°C for 45 minutes unless stated otherwise. The efficiency of plasmin generation was followed by

measuring the conversion of the single chain plasminogen molecule (Mr=90,000) into heavy chains (Mr=60,000) and light chains (Mr=25,000) as determined by the migration of radiolabeled proteins, following denaturation and reduction, on 10% SDS-polyacrylamide gels. The migration of labeled proteins was determined by autoradiographic exposure of dried gels to Kodak XAR 5 film with intensifying screens at -70°C for 20 hours.

Bacteria

The group A β-hemolytic streptococcal strain 64 had been previously subjected to mouse passage as described by Reis et al., (1984). The parent strain (64/P), as well as strains isolated after three (64/3) and fourteen (64/14) mouse passages, were grown in either Todd-Hewitt broth (DIFCO, Detroit, Mich.) or chemically defined media (Van De Rijn and Kessler, 1980) overnight at 37°C as stationary cultures (Yarnall and Boyle, 1986b). The bacteria were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween-20 and 0.02% sodium azide. The bacteria were heat killed at 80° C for 10 minutes, a treatment that did not alter their plasmin binding potential, but eliminated the production of soluble plasminogen activators which would interfere with these studies. The suspension was centrifuged, the pellet washed twice with PBS and then resuspended at 10% wet weight/volume in PBS containing 0.05% Tween-20 and 0.02% sodium azide. Samples were stored at -20°C. The concentration of a bacterial suspension was determined by counting bacterial chains in a Neubauer hemacytometer (Fisher Scientific, Orlando, FL).

Determination of Binding of Radiolabeled Proteins to Bacteria

The light scatter at 550 nm was determined to standardize the concentration of organisms used in subsequent tests. A light scatter

reading of 0.3 corresponded to approximately 2 x 10^9 organisms/ml (Yarnall and Boyle, 1986b). A standardized number of bacteria (approximately 10^9 organisms) were incubated with labeled proteins (approximately 30,000 cpm/tube) in a total volume of 400 μl of VBS-gel for 1 hour at 37°C. The bacteria were pelleted by centrifugation at 1000 x g for 10 minutes and washed twice with 2.0 ml VBS-gel. The radioactivity associated with the bacteria was determined in a Beckman 5500 autogamma counter. All estimates were carried out in duplicate.

Fibrin Plate Assay

Fibrin plates were prepared using 5 cm diameter disposable petri dishes. Ten ml of 0.1% fibrinogen in PBS were clotted with 0.2 ml of bovine thrombin (10 NIH u/ml) in 0.5 M $CaCl_2$. Twenty pmoles of plasmin were bound to 100 μl of a 10% w/v solution of the group A streptococcus, 64/14, in a total volume of 400 μl of VBS-gel. The mixture was incubated at 37°C for 45 minutes. A parallel series of samples containing bacteria with no added plasmin served as the negative control. Fifty μl of a suspension of bacteria or bacteria plus plasmin were placed either directly onto a fibrin plate or onto a 0.22 μm Millipore filter placed between the bacteria and the fibrin plate. The plates were incubated for 20 hours at 37°C and the degree of hydrolysis was scored by measuring the area of the zone of clearing from the underside of the plate. In each experiment a control of free plasmin was included and each estimate was carried out in duplicate.

Plasmin-Inhibitor Complex Generation

Plasmin was generated from plasminogen as described above. Three 130 μl aliquots of the labeled enzyme were placed into separate microtubes (approximately 2 x 10^6 cpm/tube), and incubated with a 200-

fold molar excess of either PPACK, aprotinin, or pNpGB for 10 minutes at room temperature. The volume of each sample was increased by the addition of 200 μl of VBS-gel, and each was applied to a separate G-25 column (PD 10 Pharmacia Fine Chemical) to remove free inhibitor. Five hundred μl fractions were collected and counted in an autogamma counter to localize the modified ^{125}I plasmin. Fractions containing the labeled protein-inhibitor complexes were pooled. Aliquots of the three labeled complexes were mixed with a 10-fold molar excess of α_2-AP in a final reaction volume of 400 μl for 10 minutes at room temperature. Plasmin and plasminogen were included as controls. The volumes of each solution were adjusted to 1.1 ml with VBS-gel and then used in a direct binding assay to group A streptococci. Each of the plasmin-inhibitor samples that has been incubated with excess α_2-AP was monitored on non-reducing SDS-polyacrylamide gels as described by Weber and Osborn (1969) for the formation of a high molecular weight complex.

Determination of Plasmin Activity While Bound to Bacteria

To five microtubes, each containing 100 μl of a 10% w/v solution of the group A streptococcus, 64/14, in a total volume of 400 μl VBS-gel, 10 nM plasmin was added and allowed to bind for 40 minutes at 37°C. Five other tubes containing plasmin but no bacteria and one tube containing bacteria alone were prepared as controls. The bacteria were pelleted and washed twice with 1.0 ml VBS-gel and resuspended in 400 μl VBS-gel containing a 10-fold molar excess of either pNpGB, PPACK, aprotinin, α_2-AP or buffer alone. All samples were then incubated for 15 minutes at room temperature. The samples containing bacteria were pelleted by centrifugation, washed with 1.0 ml of VBS-gel and resuspended with vigorous vortexing in 400 μl VBS-gel.

To each tube, 20 μl of an 8.0 mM solution of the chromogenic substrate, H-D-val-leu-lys-paranitroanilide, was added,to yield a final concentration of substrate in the reaction mixture of 400 μM. The tubes were mixed by vortexing and incubated at 37°C for 25 minutes. At that time the enzyme reaction was quenched by the addition of 400 μl of 10% (v/v) acetic acid, the samples were then centrifuged for 5 minutes at 10,000 x g and the optical densities of the solutions at 405 nm were determined. The release of paranitroaniline from the synthetic substrate monitored at this wavelength was directly proportional to the enzymatic activity of plasmin. Control samples of substrate alone and substrate plus bacteria were included and each estimate was carried out in duplicate.

Effect of pH on Plasmin Binding to Bacteria

To assess the effect of pH on the bacterium:plasmin(ogen) interaction, 50 μl of labeled plasminogen or plasmin (approximately 2 x 10^5 cpm) were added to 1.0 ml of VBS containing 0.05% Tween-20 adjusted to the appropriate pH. After 15 minutes at room temperature, 50 μl of VBS containing approximately 10^7 bacteria (strain 64/14) were added and the mixture was incubated at 37° C for 15 minutes. The bacterial suspensions were centrifuged at 1,000 x g for 7 minutes to separate bacteria from unbound labeled proteins and the pellets were washed twice with 2.0 ml of VBS at the appropriate pH. The radioactivity associated with the bacterial pellet in duplicate experiments was measured using a Beckman 5500 autogamma counter.

To assess the effect of ionic strength on the bacterium-plasmin(ogen) interaction, similar studies were carried out in solutions containing different concentrations of NaCl with 0.05% Tween-20. The

bacterial pellets were washed in the appropriate NaCl concentration to remove unbound labeled proteins.

Effect of Ca^{++} and Mg^{++} on Plasmin Binding

Binding of radiolabeled plasmin to group A streptococci strain 64/14 was studied in the following buffers: 1) VBS-gel containing 0.00015 M Ca^{++} and 0.001 M Mg^{++}, or 2) metal free VBS-gel containing 0.15 M EDTA. In each case 400 μl of buffer were added to 100 μl of VBS-gel containing approximately 10^7 bacteria and 100 μl of VBS-gel containing 3 x 10^5 cpm of radiolabeled plasmin. After incubation at 37° C for 15 minutes, the mixtures were centrifuged at 1,000 x g for 7 minutes to separate bacteria from unbound radiolabel, the pellets were washed twice with 2.0 ml of the appropriate buffer, and radioactivity associated with the bacterial pellet in duplicate tubes was measured.

Inhibition of Binding of Plasmin by Amino Acids

Labeled plasmin (100 μl containing approximately 2 x 10^5 cpm) was added to 200 μl VBS-gel containing varying concentrations of epsilon-aminocaproic acid (EACA), lysine, or arginine, and incubated at 37° C for 15 minutes. The pH of each solution was 7.0. One hundred μl of VBS-gel containing 10^7 bacteria (strain 64/14) were then added and the mixture was incubated at 37°C for 15 minutes. The bacterial suspensions were centrifuged at 1,000 x g for 7 minutes and washed twice with 2.0 ml of VBS-gel containing the same concentration of amino acid present during the incubation period. The percent inhibition of binding was calculated for duplicate samples by comparison with binding in VBS-gel alone.

The ability of EACA, lysine, or arginine to dissociate bound plasmin from the bacteria was examined in the following manner. Labeled plasmin was incubated with 10^7 bacteria in VBS-gel at 37° C for 15 minutes. The

bacteria were pelleted by centrifugation and washed twice with 2.0 ml of VBS-gel. After determining the radioactivity associated with the bacteria, the pellets were resuspended in solutions of VBS-gel containing varying concentrations of amino acid or amino acid analogs (pH 7.0) as described above. The mixtures were incubated at 37° C for 15 minutes and washed twice with VBS-gel containing the appropriate amino acid concentration. The radioactivity associated with the bacteria in duplicate samples was again measured and the percentage dissociated was calculated.

Determination of K_D and Receptor Density

Labeled plasmin (25,000 to 250,000 cpm) in 100 μl of VBS-gel was added to 3 x 10^6 bacteria in 300 μl of VBS-gel, pH 7.4, and incubated at 37°C for 15 minutes. The bacterial suspensions were centrifuged at 1,000 x g for 10 minutes and washed twice with 2.0 ml of VBS-gel. All determinations were performed in triplicate. Total binding was determined by measuring the radioactivity associated with the bacterial pellet when only labeled plasmin was offered. Non-specific binding was determined by pre-incubating bacteria at 37° C for 15 minutes in VBS-gel, pH 7.4, containing unlabeled plasmin at a 100-fold molar excess of the labeled plasmin. Specific binding was calculated by subtracting non-specific binding from total binding for each amount of labeled plasmin offered. The amount of free labeled plasmin was calculated by subtracting the amount of specifically bound labeled plasmin from the total amount of labeled plasmin offered.

The apparent dissociation constant (K_D) was determined by two methods. A non-linear least squares analysis of the total counts offered vs. the counts bound fit to the simple Michaelis-Menten equation was

performed as described by Lottenberg et al., (1985). The concentration of plasmin was determined by converting counts per minute to moles using the known specific activity for the labeled plasminogen. Scatchard analysis (Scatchard, 1949) of these data was also performed as described by Lottenberg et al., (1987). Counts bound vs. counts bound/counts free was plotted and the slope (representing $-1/K_D$) was determined by linear regression. The X-intercept (counts bound) was converted to moles of plasmin. To determine the receptor densities the number of moles of plasmin bound was determined by extrapolating the Scatchard plot and determining the intercept. This represented the maximal binding of plasmin to a known number of bacteria (derived by hemacytometer chamber counts).

Plasmin which had been bound to and eluted from strain 64/14 by treatment with lysine was also examined in similar binding studies. Eluted plasmin was obtained by incubating 2.0 ml of stock 10% wet weight/volume bacterial suspension (strain 64/14) with approximately 20 μg of labeled plasmin at room temperature for 45 minutes. This suspension was centrifuged at 1,000 x g for 10 minutes and washed once with 10 ml of VBS-gel, and the radioactivity associated with the bacterial pellet was measured. The pellet was then resuspended in VBS-gel containing 20 mM lysine and incubated at room temperature for 30 minutes. The suspension was centrifuged and the supernatant recovered. Approximately 90% of the radioactivity originally associated with the bacterial pellet was dissociated by the lysine treatment. The dissociated plasmin in the supernatant was then subjected to gel filtration on a G-25 column to separate lysine from plasmin. Fractions containing plasmin were collected and stored at -20°C.

Results

Twenty β hemolytic streptococcal isolates were grown overnight at 37°C and tested for their ability to bind radiolabeled plasminogen, plasmin, urokinase, or trypsin as described in the Methods Section. The results (see Table 2-1) showed that all twenty group A isolates bound plasmin but failed to bind significant quantities of plasminogen or any of the other labeled proteins, i.e. less than 10% of the offered label. Furthermore, the expression of plasmin binding ability was shown to be present on bacteria grown in either Todd-Hewitt broth or chemically defined media (data not shown). Plasmin binding was found to be dependent on the concentration of bacteria and was maximal within two minutes at 37°C. Pre-incubation with excess unlabeled plasmin prevented binding of the labeled plasmin. In the absence of unlabeled plasmin, strain 64/14 consistently bound approximately 60% of the radioactive plasmin offered and was used to analyze further the selective plasmin binding activity. In my initial attempts to characterize the differential binding of plasminogen and plasmin to a group A streptococcus I compared the kinetics of generation of plasmin from plasminogen with the ability of labeled protein to bind to the bacteria. Conversion of plasminogen to plasmin occurs when a single arginine-valine bond is split in the zymogen by action of the enzyme urokinase (Groskopf et al., 1969). The zymogen activation reaction can be monitored on SDS-polyacrylamide gels following reduction of disulfide bonds.

The zymogen molecule migrates as a single polypeptide chain with a M_r of approximately 90,000 daltons. The active enzyme plasmin migrates under these conditions as two distinct polypeptide chains (a heavy chain with an M_r of approximately 60,000 daltons and a light chain with an M_r

Table 2-1.

Binding of radiolabeled proteins to various nephritogenic and non-nephritogenic group A streptococci.

STRAIN	M-TYPE	PLASMINOGEN	PLASMIN	UROKINASE	TRYPSIN
A992*	18	-	+	-	-
B923	12	-	+	-	-
D897*	12	-	+	-	-
B512	4	-	+	-	-
B438	18	-	+	-	-
B512	NT	-	+	-	-
A928	55	-	+	-	-
64/14	NT	-	++	-	-
B905	2	-	+	-	-
B281	12	-	+	-	-
B920	49	-	++	-	-
B915	49	-	+	-	-
A374	12	-	+	-	-
B931*	2	-	+	-	-
A207	2	-	+	-	-
F2030	1	-	+	-	-
A547	NT	-	+	-	-
64/P	NT	-	++	-	-
648	1	-	+	-	-
A995	57	-	+	-	-

* Non-nephritis causing strains
- = Less than 10% bound of total counts offered
+ = 10% to 30% bound of total counts offered
++ = Greater than 30% bound of total counts offered
Approximately 3 X 10^8 bacteria/tube heat killed at 80°C for 10 min.
NT = Not typable

of approximately 25,000 daltons). The activation reaction can be stopped at any time by addition of a 10-fold molar excess of p-nitrophenyl p-guanidinobenzoate (pNpGB). Consequently, it is possible to obtain plasminogen in various stages of activation and compare the ability of the labeled proteins to bind to a group A streptococcus. The results presented in Figure 2-1, panel A demonstrate that the activation of plasminogen to plasmin could be readily monitored. As the conversion of plasminogen to plasmin proceeded, an increase in the binding of labeled protein occurred which correlated with the concentration of plasmin in the reaction mixture (Figure 2-1, panel B).

The conversion of plasminogen to plasmin yields a serine active site that is not expressed in the zymogen. In the next series of experiments the role of the active site in binding of the enzyme to the bacteria was assessed. Plasmin was treated with the active site titrant pNpGB, the small naturally occurring inhibitor aprotinin (Fritz and Wunderer, 1983), the selective histidine modifying agent, phe-pro-arg chloromethyl ketone [PPACK] (Kettner et al., 1980), and the physiological regulator α_2 antiplasmin (α_2-AP) (Mori and Aoki, 1976). The ability of the various inhibited forms of plasmin to bind to a group A streptococcus was measured. The results presented in Figure 2-2 demonstrate that plasmin treated with pNpGB, aprotinin, or PPACK were all capable of binding to the bacteria in the presence of α_2-AP. By contrast unmodified plasmin incubated with the physiological inhibitor, α_2-AP, failed to bind. Each of the plasmin-inhibitor samples that had been incubated with excess α_2-AP was monitored on non-reducing SDS-polyacrylamide gels for the formation of a high molecular weight complex. The high molecular weight band observed in the third lane indicates the formation of a stable

Figure 2-1. Binding of plasmin to bacteria: comparison of the kinetics of generation of plasmin and its ability to bind to the group A streptococcus 64/14: Labeled plasminogen was converted to plasmin by treatment with urokinase. The kinetics of generation of plasmin was monitored on SDS-polyacrylamide gels under reducing conditions. The conversion of single chain, high molecular weight, plasminogen (Mr approximately 90,000) into heavy (Mr approximately 60,000) and light chains (Mr approximately 25,000) of plasmin was monitored. At each time point the ability of labeled proteins to bind to the group A streptococcus 64/14 was measured as described in the Methods. The data are presented as the mean ± the standard deviation of duplicate experiments.

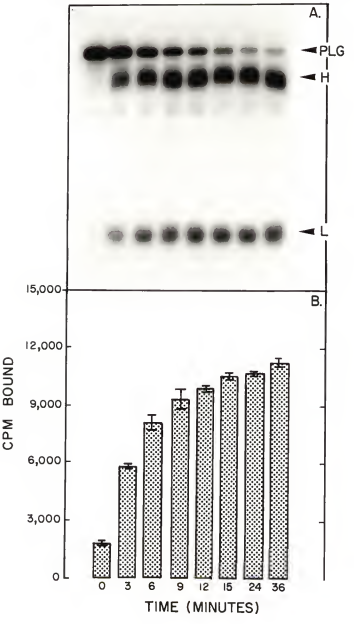
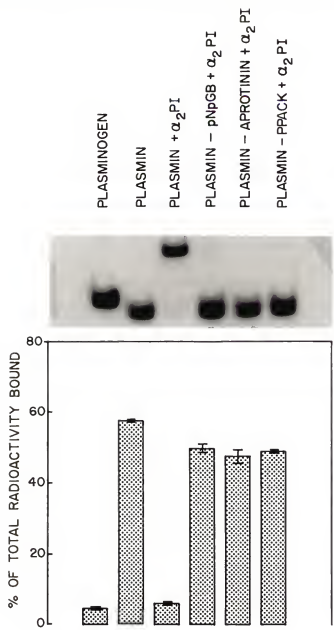

A.
PLG
H
L
B.
15,000
12,000
9,000
6,000
3,000
0
CPM BOUND
0
3
6
9
12
15
24
36
TIME (MINUTES)

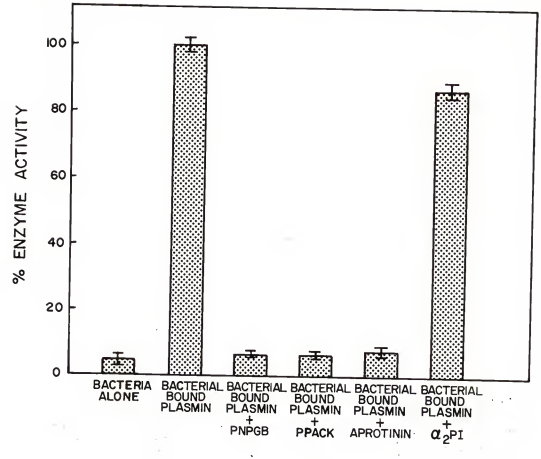
Figure 2-2. Effect of inhibiting the active site of plasmin on its ability to bind to the group A streptococcal strain 64/14: The lower panel demonstrates the binding of the group A streptococcus to labeled plasminogen, plasmin, plasmin pretreated with excess α_2PI, plasmin treated with excess pNpGB, plasmin treated with excess aprotinin or plasmin treated with excess phe-pro-arg chloromethyl ketone,[PPACK], as described in the Methods. The data are presented as the mean ± the standard deviation of duplicate experiments. The upper panel demonstrates the analysis of each of the plasmin-inhibitor samples that had been incubated with excess α_2PI. Samples were monitored on non-reducing SDS-polyacrylamide gels for the formation of a high molecular weight complex.


PLASMINOGEN
PLASMIN
PLASMIN + α_2PI
PLASMIN – pNpGB + α_2PI
PLASMIN – APROTININ + α_2PI
PLASMIN – PPACK + α_2PI
% OF TOTAL RADIOACTIVITY BOUND
0
20
40
60
80

complex of plasmin with its physiological inhibitor α_2-AP (Figure 2-2, upper panel). Pretreatment of plasmin with aprotinin, pNpGB, or PPACK inhibited the ability of the enzyme to form the covalent linkage inhibition reaction with α_2-AP demonstrating that under the experimental conditions used the plasmin active site was modified.

The next series of experiments were designed to determine whether bacterial bound plasmin was capable of retaining its enzymatic activity. Radiolabeled plasmin was generated and incubated with a suspension of group A streptococci for 40 minutes at 37°C. The bacteria with the associated plasmin were recovered by centrifugation, washed twice with buffer, and then tested for their ability to cleave the chromogenic synthetic substrate H-D-val-leu-lys-paranitroanilide (as described in the Methods). In these experiments a control of bacteria alone failed to hydrolyze the chromogenic substrate, while bacteria pre-incubated with plasmin were found to cleave the substrate efficiently. The ability of bacterial bound plasmin to be affected by a variety of different inhibitors was tested. The results in Figure 2-3 demonstrate that addition of pNpGB, PPACK, or aprotinin to the bacterial bound enzyme was capable of inhibiting its enzyme activity for the synthetic substrate. By contrast, addition of α_2-AP failed to reduce the enzyme activity (Figure 2-3). All inhibitors were used in excess of that required to totally inhibit an equivalent concentration of plasmin in the fluid phase. Since α_2-AP failed to regulate the bacterial bound enzyme, one might predict that the large molecule fibrin, the natural substrate of plasmin, would also be prevented from occupying the substrate pocket in the active site. To test this prediction, bacteria with plasmin bound to their surface were placed on a fibrin plate and their ability to

Figure 2-3. Regulation of bacterial bound enzyme activity by a variety of different serine protease inhibitors: Bacterial pellets were pre-incubated with plasmin, washed and resuspended in buffer containing excess pNpGB, PPACK, aprotinin, α_2-AP or buffer alone for 15 minutes at room temperature. Following incubation with the inhibitor the bacteria were pelleted and washed. Enzyme activity was then measured by the ability of the samples to hydrolyze the chromogenic substrate HD-Val-leu-gly-paranitroanilide as described in the Methods. The data are presented as the mean ± the standard deviation of duplicate experiments. The hydrolysis by bacterial bound plasmin in the absence of any inhibitor represents 100% activity.


% ENZYME ACTIVITY
100
80
60
40
20
0
BACTERIA ALONE
BACTERIAL BOUND PLASMIN
BACTERIAL BOUND PLASMIN + PNPGB
BACTERIAL BOUND PLASMIN + PPACK
BACTERIAL BOUND PLASMIN + APROTININ
BACTERIAL BOUND PLASMIN + α_2PI

mediate dissolution of the fibrin clot was measured. The results presented in Table 2-2 demonstrate that the bacterial bound plasmin still retained its ability to cleave fibrin. These effects could not be accounted for by dissociation of plasmin from the bacteria, since clot lysis did not occur when the microbe-plasmin complex was separated from the clot by a 0.22 μm Millipore filter (Table 2-2). Under these experimental conditions, unbound plasmin was capable of passing through the filter and causing fibrin degradation.

The following series of experiments, which characterize further the interaction of human plasmin and this group A streptococci bacteria were performed by Dr. Tim A. Broeseker, a Fellow in the department of medicine, division of hematology at the University of Florida.

In his initial experiments the binding of labeled plasmin or plasminogen to the group A streptococcal strain 64/14 as a function of pH was tested. Labeled proteins were pre-equilibrated in VBS-gel buffers of differing pH's before the addition of bacteria. After an incubation period of 15 minutes at 37°C, the radioactivity associated with the bacteria was measured by pelleting the micro-organisms and washing free the unbound label with buffer of the appropriate pH as described in the Methods. Maximal binding of plasmin to the bacteria was observed between pH 5 and 8 with approximately 60% of counts offered being bound by the group A streptococcus 64/14, (Figure 2-4). In contrast, addition of labeled plasminogen to the bacteria over the entire pH range tested (pH 5-9) resulted in direct binding of less than 10% of offered counts. (Figure 2-4). Similar studies were carried out to determine the effect of ionic strength on binding of radiolabeled plasmin and plasminogen to the group A streptococcal strain 64/14. Labeled proteins were pre-

Table 2-2.

Ability of bacterial bound plasmin to solubilize a fibrin clot.

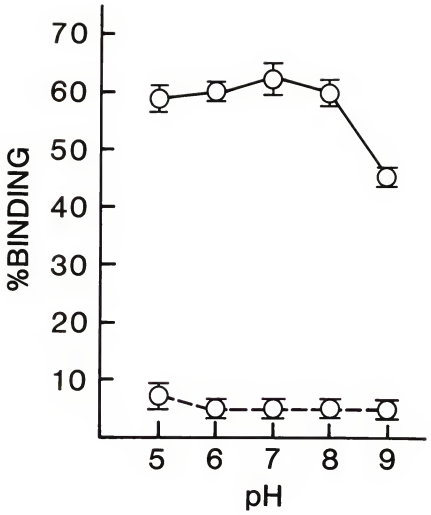
Sample	Hydrolysis of Fibrin[a]	
	Direct[b]	Indirect[c]
Bacteria Alone	-	-
Bacterial Bound Plasmin	++	-
Plasmin Alone	+++	+++

[a]Under the experimental conditions described in the Methods section, a (+++) reaction represented a zone of clearing with a diameter of 1.0-1.5 cm, a (++) reaction represented a zone of clearing of 0.5-1.0 cm, a (+) reaction represented a zone of clearing from 0-0.5 cm, and (-) represents no clearing.

[b]Sample placed directly onto a fibrin plate.

[c]Sample placed onto a filter placed between the bacteria and the fibrin plate.

Figure 2-4. Binding of ^{125}I-plasmin or ^{125}I-plasminogen to the group A streptococcal strain 64/14 as a function of pH: The data are presented as the mean ± the standard deviation. Measurements of duplicate experiments were performed and are expressed as the percent of total counts offered (20,000 cpm) which were associated with the bacterial pellet. (O———O) ^{125}I-plasmin; (O-----O) ^{125}I-plasminogen.

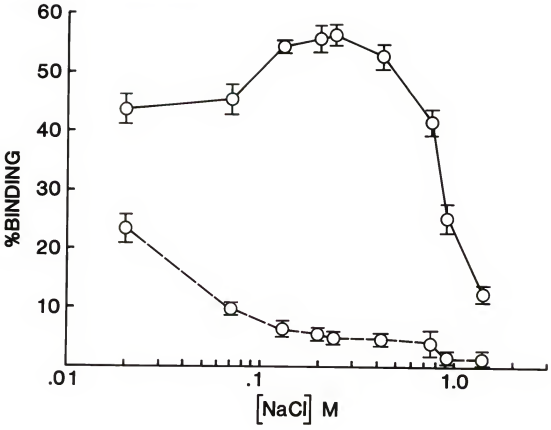

70
60
50
40
30
20
10
%BINDING
5
6
7
8
9
pH

equilibrated in NaCl solutions of varying ionic strength before the addition of bacteria. Following an incubation period of 15 minutes at 37°C the bacteria were washed with solutions containing the appropriate concentration of NaCl and the number of counts associated with the bacteria determined. The results in Figure 2-5 demonstrate that plasmin binding was dependent on ionic strength and that optimal binding occurred between 0.1 and 0.4 M NaCl. In this range of salt concentrations, less than 10% of plasminogen bound to bacteria. As the ionic strength was lowered below 0.075 M NaCl, significant binding of plasminogen to the bacteria was observed.

Binding of labeled plasmin to the group A streptococcal strain 64/14 was examined in the presence and absence of divalent cations to determine if these metal ions were important for plasmin binding. Binding studies were carried out in VBS-gel at pH 7.4 containing 0.00015 M Ca^{++} and 0.001 M Mg^{++} or in metal free VBS-EDTA-gel at pH 7.4. After incubation at 37°C for 15 minutes, the bacteria were washed twice with the appropriate buffer and radioactivity associated with the bacterial pellets was measured. The amount of plasmin bound by the bacteria was the same in the presence or absence of divalent cations. (data not shown).

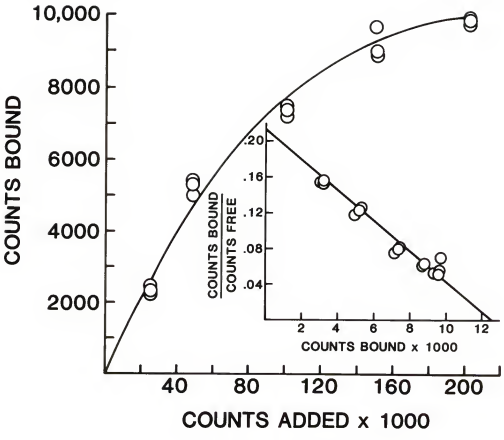
After identification of the optimal binding conditions for the plasmin:bacterium interaction, the affinity of the plasmin receptor for its ligand was determined in 0.15 M VBS-gel buffer at pH 7.4. In the initial studies the group A β-hemolytic strain 64/14 was used. Preliminary kinetic studies were conducted to establish first that equilibrium between bacterial bound and free plasmin had been achieved, and second the conditions under which saturation of bacterial plasmin receptors could be demonstrated. Binding equilibrium was found to be

Figure 2-5. Binding of ^{125}I-plasmin or ^{125}I-plasminogen to the group A streptococcal strain 64/14 as a function of ionic strength: The data are presented as the mean ± the standard deviation. Measurements of duplicate experiments were performed and are expressed as the percent of total counts offered (20,000 cpm) which were associated with the bacterial pellet. (o———o) ^{125}I-plasmin; (o-----o) ^{125}I-plasminogen


60
50
40
30
20
10
%BINDING
.01
.1
1.0
[NaCl] M

established within 15 minutes at 37°C. Under these conditions using 10^6 bacteria and increasing concentrations of labeled plasmin, it was possible to demonstrate a plateau in plasmin binding capacity consistent with saturation of bacterial receptors, as shown in Figure 2-6. To correct for non-specific binding of radiolabel, similar studies were carried out in which binding of radiolabeled plasmin to the bacteria was measured in the presence of 100-fold molar excess of unlabeled plasmin. Non-specific binding demonstrated a linear relationship to counts offered and was less than 5% in all tubes (data not shown). Analysis of this data by least squares and Scatchard analysis demonstrated a K_D of approximately 5×10^{-11} M for the association of plasmin with its receptor on the mouse passaged group A streptococcus strain 64/14. Scatchard analysis of the binding data indicates that there is a single population of plasmin receptors on streptococci (see inset, Figure 2-6), and that strain 64/14 possesses approximately 800 receptors per bacterium. Similar studies were carried out with the group A strain 64/P (the original isolate that was used in the previous mouse passage studies (Reis et al., 1984)) and with strain 64/3 isolated following three passages of strain 64 in mice. The 64/3 strain was less virulent in mice than 64/P which in turn was much less virulent than the strain recovered after 14 mouse passages, 64/14 (Reis et al., 1984). The 64/P and 64/3 strains were studied using the same protocol described for the generation of the data using strain 64/14. The results indicated that the plasmin receptor on 64/P had a K_D of approximately 1×10^{-10} M and each bacterium expressed approximately 200 receptors. The 64/3 strain displayed a plasmin receptor with a K_D of approximately 6×10^{-10} M and approximately 3,500 receptor sites per bacterium. Analysis of the

Figure 2-6. Specific binding of ^{125}I-plasmin to 10^7 group A streptococci, strain 64/14, following a 15 minute incubation at 37°C in VBS-gel at pH 7.4: Measurements of triplicate experiments were performed. Specific binding was determined as described in Methods. The inset represents the Scatchard analysis of the specific binding data.


10,000
8000
6000
4000
2000
COUNTS BOUND
40
80
120
160
200
COUNTS ADDED x 1000
.20
.16
.12
.08
.04
COUNTS BOUND / COUNTS FREE
2
4
6
8
10
12
COUNTS BOUND x 1000

Scatchard plots of 64/P and 64/3, like that shown for 64/14 in Figure 2-6, demonstrated only a single class of plasmin receptors expressed on these bacteria (Broeseker et al., 1988). Plasmin(ogen) contains lysine binding sites which also bind analogous amino acids (Winn et al., 1980). Epsilon-aminocaproic acid (EACA) approximates the side chain structure of lysyl residues incorporated in intact proteins and has higher affinity than lysine for these sites on plasmin(ogen), whereas arginine binds with lower affinity (Winn et al., 1980). In order to assess the possible role of the lysine binding sites of plasmin in its interaction with the bacterial receptor, the binding of plasmin to the group A streptococcus strain 64/14 in the presence of EACA, lysine, or arginine was determined. Binding was measured in 0.15 M VBS-gel, pH 7.4, containing amino acid in increasing concentrations. The percentage inhibition of binding was determined by comparison with the binding in VBS-gel pH, 7.4, buffer alone. The results of these studies are presented in Figure 2-7 and demonstrate that binding of plasmin to the group A streptococcus 64/14 was inhibited by each amino acid in a concentration dependent fashion. Fifty percent inhibition of binding of plasmin to the bacteria was observed at an EACA concentration of 0.15 mM, a lysine concentration of 2.0 mM, and an arginine concentration of 25 mM. In similar studies, plasmin was pre-bound to the group A streptococcus and a concentration dependent elution of bound radiolabel was observed on incubation with EACA, lysine, or arginine (Figure 2-8). The concentration of amino acid required to elute 50% of the bound plasmin was approximately equivalent to that required to inhibit plasmin binding by 50% (compare Figures 2-7 and 2-8).

Figure 2-7. Inhibition of binding of ^{125}I-plasmin to the group A streptococcal strain 64/14 in VBS-gel containing various concentrations of epsilon-aminocaproic acid, lysine, and arginine: Measurements of duplicate experiments were performed and the data are presented as the mean ± the standard deviation. The percent inhibition of binding was calculated by comparing with binding in VBS-gel alone. (O———O) epsilon- aminocaproic acid; (O-----O) lysine; (O-•-•-O) arginine.

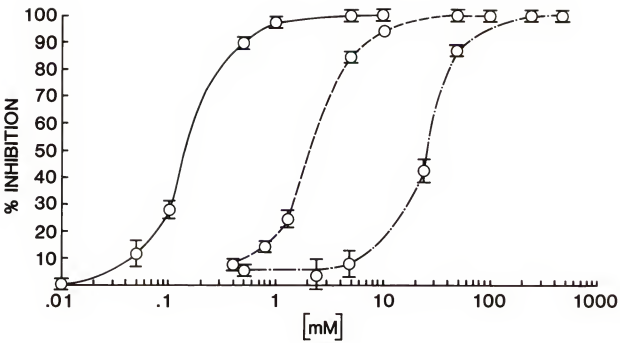
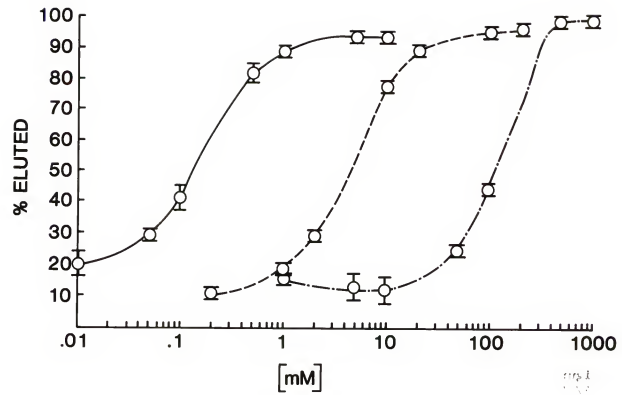

% INHIBITION
100
90
80
70
60
50
40
30
20
10
.01
.1
1
10
100
1000
[mM]

Figure 2-8. Elution of ^{125}I-plasmin from group A streptococcal strain 64/14 in VBS-gel containing various concentrations of epsilon-aminocaproic acid, lysine, and arginine: Measurements of duplicate experiments were performed and the data are presented as the mean ± the standard deviation. Percent eluted was calculated by comparing the radioactivity associated with the bacterial pellet before and after incubation in the given amino acid solution. (O———O) epsilon-aminocaproic acid; (O-----O) lysine; (O-•-•-O) arginine.


% ELUTED
100
90
80
70
60
50
40
30
20
10
.01
.1
1
10
100
1000
[mM]

Discussion

Plasminogen, an inactive zymogen can be converted to the protease plasmin by a variety of plasminogen activators (Collen, 1980). This enzyme demonstrates broad substrate specificity. In addition to fibrin cleavage, plasmin can activate the first component of the classical complement pathway, hydrolyze coagulation factors, degrade components of basement membrane, and break down connective tissue (Atichartakarn et al., 1978; Jones and DeClerck, 1980; Liotta et al., 1981). Furthermore a variety of potent split products are generated as a consequence of plasmin activity, e.g. chemotactic fibrinopeptides (Kay et al., 1974). Effective regulation of plasmin activity is therefore important in order to prevent tissue damage and inflammation. Normally the selective protease inhibitor α_2-antiplasmin regulates plasmin activity in man (Aoki et al., 1977).

Interaction of streptococci and streptococcal products with the fibrinolytic system has been recognized for many years (Tillett and Sherry, 1949). The observation that certain streptococci could lyse a fibrin clot lead to the identification and isolation of streptokinase. This secreted protein is known to bind to human plasminogen and plasmin with a similar affinity (Reddey and Markus, 1972). In this study I have identified a surface receptor on certain group A streptococci, grown in either Todd-Hewitt broth or chemically defined media, that specifically binds to plasmin while demonstrating no significant affinity for the zymogen form of the molecule, plasminogen. Thus the surface receptor we have identified is distinct from streptokinase. Furthermore, this binding phenomenon did not appear to be simply a function of the ligand being a serine protease, since no binding activity was demonstrated with the two other serine proteases examined, trypsin and urokinase.

Binding of plasmin to its bacterial receptor does not inhibit the ability of the enzyme to cleave either small synthetic substrates or its natural substrate fibrin. Aprotinin, a naturally occurring tight-binding inhibitor of plasmin and phe-pro-arg chloromethylketone which chemically modifies the histidine residue of the active site can react with the bound plasmin and neutralize its enzymatic activity. These findings suggest that the catalytic portion of the plasmin molecule is not interfered with by the association with the bacteria. Of interest was the observation that the enzymatic activity of bacterial bound plasmin could not be regulated by addition of its specific inhibitor, α_2-antiplasmin. Alpha$_2$-antiplasmin is a potent inhibitor of plasmin in the fluid phase forming a 1:1 stoichiometric complex between the enzyme and inhibitor.

The failure of α_2-antiplasmin to regulate bacterial bound plasmin provides the bacteria with a potential mechanism for tissue invasion by virtue of the ability of plasmin to hydrolyze components of connective tissue and basement membranes. Recent studies of the invasive characteristics and metastatic potential of tumor cells has suggested a key role for plasminogen activators in this process (Dano et al., 1985). The ability of certain group A streptococci to produce a plasminogen activator (e.g., streptokinase) and also to express a receptor for the activation product plasmin may account for certain of its invasive properties. Furthermore, since plasmin bound to a group A streptococcus is incapable of inhibition by α_2-antiplasmin the bacteria has associated with it a non-regulatable proteolytic activity that may help to contribute to its tissue invasive properties.

A variety of receptors for human proteins have been described on streptococci. These include receptors for key components of the immune

system such as C1q (Yarnall et al., 1986), IgG (Kronvall, 1973), and IgA (Russell-Jones et al., 1984), the serum protein fibrinogen (Kronvall et al., 1979), and the stromal structural proteins laminin (Switalski et al., 1984), and fibronectin (Myhre and Kuusela, 1983). The importance of any of these receptors in the pathogenic process remains controversial. Our recent observation that streptococci also have a receptor that is specific for human plasmin adds to this list (Lottenberg et al., 1987). Although the primary substrate for plasmin is fibrin, plasmin is a non-specific protease capable of also hydrolyzing such extracellular matrix proteins as thrombospondin, fibronectin, and laminin, while also exposing matrix components for degradation by other enzymes (Knudsen et al., 1986). The ability to bind plasmin in an active form which can not be regulated by its efficient physiological regulator α_2-antiplasmin could provide for surface mediated protease activity and a mechanism for tissue invasiveness by plasmin receptor-positive bacteria (Lottenberg et al., 1987).

In this study it was shown that the group A streptococcus strain 64/14 demonstrates optimal binding of its ligand at physiological pH and ionic strength. The interaction had a high affinity ($K_D = 5 \times 10^{-11}$ M) and demonstrated a linear Scatchard plot indicating that a single population of plasmin receptors was present on the bacteria (Broeseker et al., 1988). There was no evidence for either an additional low affinity receptor or for any cooperativity, positive or negative, in the binding of ligand with the specific receptor. In agreement with our previous observations (Lottenberg et al., 1987), there was no evidence for specific interaction between bacteria and the native zymogen form of the protein, plasminogen.

Plasmin(ogen) has several lysine binding sites located on its heavy chain. The low affinity sites are primarily important for binding to fibrin and the high affinity site is important for the interaction with α_2-antiplasmin (Wiman et al., 1979). In order to assess the possible role of these lysine binding sites in the interaction between plasmin and its bacterial receptor, the bacterial binding of plasmin in the presence of increasing concentrations of EACA, lysine, or arginine. The results in Figure 2-7 and 2-8 demonstrate that binding could be inhibited, and bound plasmin could be eluted, by these amino acids in a concentration-dependent manner. Eluted plasmin could be re-bound to bacteria simply by removing lysine from the eluted plasmin solution, indicating a possible importance of the lysine binding sites for the receptor:plasmin interaction. There are 4 or 5 low affinity sites (K_D = 5mM) and one high affinity site (K_D = 9μM) for EACA (Markus et al., 1978). Lysine and arginine bind to the high affinity site less tightly than does the lysine analog EACA (Wiman and Collen, 1978). Analysis of the inhibition curves for EACA, lysine, and arginine reveal that occupancy of the high affinity lysine binding site on plasmin interferes with binding to the bacteria. It is recognized that occupancy of the high affinity lysine binding site causes gross conformational changes in the plasmin(ogen) molecule, and therefore the possibility for allosteric as well as direct effects needs to be considered. The very high affinity of the receptor for plasmin, approximating the affinity of α_2-antiplasmin for plasmin ($K_D = 2 \times 10^{-10}$ M, Wiman and Collen, 1978), suggests that streptococci may be able to compete effectively with α_2-antiplasmin for plasmin. and could explain why bacterial bound plasmin cannot be regulated by α_2-antiplasmin. The enzymatic inhibition of plasmin by

α_2-antiplasmin occurs in a two-step process (Christensen and Clemmenson, 1977; Wiman and Collen, 1978). The first step is a non-covalent association of the α_2-antiplasmin molecule with the lysine binding site located in the kringle 1 region of the plasmin molecule, followed by a rapid covalent linkage to the plasmin active site serine residue. The observation that lysine or EACA effects, on the high affinity site of plasmin, disturbs the plasmin bacterial receptor interaction, together with the observation that bound plasmin is not inhibited by α_2-antiplasmin, suggests that the kringle 1 domain may be important in the binding of plasmin to bacteria, or may be inaccessible.

Previous studies have shown that passage of streptococci in mice heightened virulence with concomitant enhanced expression of certain surface proteins (Burova et al., 1980; Burova et al., 1981; Reis et al., 1984). Group A streptococcal strain 64 exhibits decreased expression of Fc receptors after 3 or 4 mouse passages (strain 64/3 and 64/4) as compared to the parent strain (64/P) (Reis et al., 1984). Following 8 mouse passages this group A streptococcus demonstrates markedly enhanced Fc receptor expression which appears to be a stable characteristic of the selected strain (Reis et al., 1984). In this study the average affinity of the plasmin receptor expressed on strains 64/P, 64/3, and 64/14 was not significantly different, indicating that mouse passage did not have a major selective pressure on the affinity of the plasmin receptor.

There was some variation in the number of plasmin receptors calculated for each bacteria. The 64/P, 64/3, and 64/14 strains displayed 200, 3500, and 800 receptor sites, respectively, per bacterium. Clearly these are average numbers of receptors per bacterium (Broeseker et al., 1988), and given the potential errors in estimating bacterial

number, the effect of phase variations in the expression of different proteins by bacteria (Cleary et al., 1987), and the heterogeneity in receptor expression among colonies (Yarnall et al., 1984), we believe that such differences need to be cautiously interpreted. It does not appear from the results of these studies that the degree of plasmin receptor expression correlates with the virulence of these group A isolates in mice. Nonetheless we calculate that binding of active plasmin in the picomolar range with any of the group A isolates studied is achievable. The high affinity for and slow off rate of bound plasmin may make these interactions with streptococci of importance in the infectious process.

The next series of studies, described in the following chapter, were designed to analyze the way in which the bacterial receptor associated with its ligand, the human plasmin molecule.

CHAPTER THREE
LOCALIZATION OF THE DOMAIN OF PLASMIN INVOLVED IN BINDING TO ITS SPECIFIC GROUP A STREPTOCOCCAL RECEPTOR

Introduction

The studies documented in Chapter Two demonstrate that certain pathogenic group A streptococci, grown in either Todd-Hewitt broth or chemically defined media, express a receptor that binds to human plasmin while demonstrating no significant reactivity with the native zymogen form of the protein, Glu-plasminogen or with other serine class proteases. Bacterial-bound plasmin retains its enzymatic activity and can no longer be regulated by its physiological inhibitor, α_2-antiplasmin. Optimal binding of plasmin to its bacterial receptor was shown to occur under physiological conditions of ionic strength and pH. This interaction of plasmin with a group A streptococcus had a high affinity with an estimated dissociation constant of approximately 1.0×10^{-10} M. Plasmin binding was inhibited reversibly by lysine or epsilon amino caproic acid, (EACA). These data suggest that the lysine binding kringle structures of the plasmin molecule might be involved in the association of plasmin with the bacterial receptor. In this chapter I describe the experiments performed to localize the region of the plasmin molecule which interacts with the bacterial plasmin receptor. Binding of plasmin to a group A streptococcus is dependent on the conformation of the plasmin molecule, and involves interactions that are distinct from those occurring between other known plasmin(ogen) binding molecules like streptokinase, fibrin, fibrinogen, thrombospondin, or α_2-antiplasmin.

Materials and Methods

Enzymes, Inhibitors and other Reagents

Urokinase and porcine elastase (type IV) were obtained from Sigma Chemical Co., St. Louis., MO. Aprotinin was obtained as Trasylol from Mobay Pharmaceuticals, New York, NY. Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem-Behring, San Diego, CA. Human Lys-plasminogen was obtained from American Diagnostica Inc., Greenwich, CT. H-D-Val-Leu-Lys-paranitroanilide (S-2251) was obtained from Helena Laboratories, Beaumont, TX.

Human Plasminogen

Native human plasminogen (Glu-plasminogen) was prepared from human plasma by chromatography on lysine-Sepharose and molecular sieving chromatography on Superose 6 (Pharmacia-FPLC, Piscataway, NJ). The purified protein appeared as a single band on a silver stain of an SDS-polyacrylamide gel. Plasminogen was quantified by measuring absorbance using a A^{280nm}_{1cm} value of 17.0 (Nilsson et al., 1982). The protein was also quantified antigenically by Laurell Rocket electrophoresis (Laurell, 1966). The purity of the isolated human plasminogen was confirmed by activation of a known quantity of plasminogen with streptokinase and measuring amidolytic activity. The observed and theoretical predicted enzymatic activity were equivalent, within experimental error. Human Lys-plasminogen, a modified form of Glu-plasminogen in which 76 of the NH_2-terminal amino acid residues are removed (Glu-1 to Lys-76) was obtained from American Diagnostica Inc., Greenwich, CT. The homogeneity of this Lys-plasminogen preparation was analyzed using both an urea gel electrophoresis procedure and an acetic acid urea gel electrophoresis procedure. This Lys-plasminogen preparation demonstrated the appropriate

migratory property (a shift to a lower M_r of approximately 85,000 daltons) in comparison to native Glu-plasminogen (M_r of approximately 92,000 daltons).

Iodination of Proteins

Glu- and Lys-plasminogen were iodinated by the chloramine T method using Iodobeads (Pierce Chem. Co., Rockford, IL) as described by Markwell (1982). The labeled proteins were separated from free iodine by passage over a G-25 column (PD-10, Pharmacia) and collected in 0.15 M Veronal buffered saline, pH 7.4, containing 0.001 M Mg^{++}, 0.00015 M Ca^{++}, and 0.1% gelatin (VBS-gel). The labeled proteins were stored in aliquots containing 0.02% sodium azide at -20°C. The concentration of ^{125}I-plasminogen was determined antigenically using a sandwich enzyme-linked immunosorbent assay (ELISA) technique utilizing goat anti-human plasminogen IgG fraction from Atlantic Antibodies, Scarborough, ME. This assay could measure plasminogen reliably in the nanogram range.

Generation of Plasmin

Lys-plasmin was generated from radiolabeled or unlabeled Glu- or Lys-plasminogen by incubation with urokinase (20 units/ml) in VBS-gel (unless stated otherwise) that contained 0.04 M lysine. The conversion of the single chain zymogen molecule to the two chain plasmin enzyme was monitored on SDS-PAGE under reducing conditions as described previously by Lottenberg et al., (1987). Conversion of the zymogen to the active enzyme was maximal after 30 min incubation at 37°C. Glu-plasmin was generated by a similar procedure with the exception that a 10-fold molar concentration of aprotinin relative to the Glu-plasminogen concentration was added prior to addition of urokinase (Swenson and Thorsen, 1981). Mini-plasmin was generated from mini-plasminogen using the same

activation procedure described to generate Lys-plasmin. Phe-pro-arg-chloromethylketone (PPACK) reacted radiolabeled or unlabeled plasmin was obtained by mixing a 5-fold molar excess of the inhibitor with plasmin and incubating at 37°C for 30 min.

Bacteria

The group A, β-hemolytic, streptococcal strain 64/14 was grown in Todd-Hewitt broth (Difco, Detroit, MI) overnight at 37°C as stationary cultures (Yarnall and Boyle, 1986a). The bacteria were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 and 0.02% sodium azide. The bacteria were heat killed at 80°C for 15 min. The suspension was centrifuged and the pellet washed twice with VBS-gel containing 0.02% sodium azide. Aliquots were stored at -20°C. Stocks of 10% wet weight/vol suspensions were prepared in VBS-gelatin containing 0.02% sodium azide. The concentration of a bacterial suspension was determined by counting bacterial chains in a Neubauer hemacytometer (Fisher Scientific, Orlando, FL).

Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out as described by Weber and Osborn (1969) with the addition of 6.0 M urea to the polyacrylamide gel. The polyacrylamide gels consisted of a 4% stacking gel layered onto a 10% or 12% polyacrylamide gel containing 0.1% sodium dodecylsulfate, 0.05 M sodium phosphate pH 7.1, 6.0 M urea. Slab gels were used in the Bio-Rad Protean II system (BioRad, Richmond, CA). Protein samples were prepared by mixing an equal volume of sample buffer containing 0.1 M sodium phosphate pH 7.1, 8.0 M Urea, and 4.0% SDS with the protein solution, and heating at 80°C for 2 minutes. Sample buffer containing 0.72 M β-mercaptoethanol was used to prepare protein samples in the reduced state.

Preparation of Elastase Digestion Fragments of Plasminogen

Elastase digestion of human plasminogen yields three defined fragments of the plasminogen molecule (Sottrup-Jensen et al., 1978). These are 1) the lysine-binding domain I (LBS-I), M_r of approx. 38,000 daltons containing kringle domains 1 through 3, 2) Lysine binding domain II (LBS-II), M_r of approx. 10-12,000 daltons consisting of the kringle domain 4, and 3) the non-lysine-binding domain known as mini-plasminogen, M_r of approx. 36,000 daltons containing the remainder of the heavy chain (kringle 5) and intact light chain. Elastase digestion was performed using established conditions (Sottrup-Jensen et al., 1978). Purified Glu-plasminogen (3.0 mg/ml) in 0.05 M Tris, 0.1 M NaCl, pH 8.0, was digested with a 40:1 molar ratio of Glu-Plasminogen to porcine elastase in the presence of 250 KIU/ml aprotinin for 6.5 hours at room temperature with gentle stirring in a total volume of 20 mls. At this time an aliquot containing 50 μg of protein was removed for analysis by SDS-PAGE and silver staining, to determine the extent of plasminogen digestion. The remainder of the reaction mixture was flash frozen and stored at -70°C. The fragments were subsequently purified by a combination of affinity chromatography on lysine-Sepharose and gel filtration on Superose 6 (Pharmacia FPLC). The concentrations of the purified proteins (see Figure 3-1, panel A) were determined spectrophotometrically, using previously reported A^{280nm}_{1cm} values of 17.0 for both Glu- and Lys-plasminogen (Holvoet et al., 1985), 14.0 for mini-plasminogen (Holvoet et al., 1985), 22.5 for LBS I (Nilsson et al., 1982), 25.0 for LBS II (Nilsson et al., 1982), and 16.0 for plasmin heavy chain and plasmin light chain (Summaria and Robbins, 1976). All proteins were aliquoted and stored at -70°C.

Preparation of Plasmin Heavy and Light Chains

Plasmin heavy and light chains were prepared essentially as described by Summaria and Robbins (1976). Twenty mg of Lys-plasmin, enzymatically inhibited with a 5 fold molar excess of aprotinin in 5 mls of 0.05 M Tris, 0.1 M NaCl, pH 8.0, was reduced by treatment with 0.1 M β-mercaptoethanol for 20 min. at 20°C. The reduced solution was then cooled in an ice slurry and carboxymethylated with 0.1 M sodium-iodoacetate on ice for 10 min. The plasmin heavy and light chains were then separated and purified by a combination of affinity chromatography on lysine-Sepharose, concentration by ammonium sulfate precipitation (4.0 g/10 ml), resuspension in 0.05 M Tris, 0.1 M NaCl, pH 8.0, and subjected to gel filtration on Superose 6 (Pharmacia FPLC). The isolated plasmin and plasminogen fragments were analyzed for purity on a reduced SDS-6 M-urea-polyacrylamide gel. As shown in Figure 3-1, panel B, the various fragments demonstrated appropriate molecular sizes and were homogeneous. Concentrations were determined as described above. All proteins were aliquoted and stored at -70°C.

Direct Binding Assay of Radiolabeled Proteins

The ability of radiolabeled plasminogen fragments to bind to the group A streptococcus 64/14 was measured as described previously by Lottenberg et al., (1987). A fixed number of bacteria were incubated with labeled proteins (approximately 30,000 cpm per tube) in a total volume of 400 μl of VBS-gel for 30 min at 37°C. The bacteria were pelleted by centrifugation at 1000 x g for 10 min and the pellets washed twice with 2.0 ml of VBS-gel. The radioactivity associated with the bacteria was determined in a Beckman 5500 Auto gamma counter (Beckman Instruments, Inc., Fullerton, CA). Non-specific background binding was

determined in replicate tubes which contained no bacteria. All estimates were performed in duplicate.

Inhibition of Plasmin Binding to Bacteria by Purified Plasmin(ogen) Fragments

The ability of different concentrations of one or more of the isolated plasminogen fragments to inhibit binding of Phe-Pro-Arg-Chloromethylketone (PPACK) reacted ^{125}I-Lys-plasmin to the group A streptococcus 64/14 was tested using a modification of the direct binding assay described above. Different concentrations of plasmin(ogen) or plasmin(ogen) fragments were mixed with a fixed dilution of a 10% w/v suspension of streptococcal strain 64/14 and PPACK reacted ^{125}I-Lys-plasmin (approx. 30,000 cpm per tube) followed by incubation for 30 min at 37°C. Bacterial associated radioactivity was determined after washing away unbound label as described above. The inhibition of binding of labeled plasmin was calculated by comparing the number of counts bound in the absence of competitor with the number of counts bound when the competitor was present. All samples were corrected for background binding of counts. Counts bound in the tubes from which bacteria were omitted or in tubes in which a 100-fold molar-excess of unlabeled ligand was added. In no case was the background level of radioactivity greater than 5% of the counts offered. Furthermore, background levels in the presence of excess cold competitor, or in the absence of bacteria were not significantly different.

Elution and Analysis of ^{125}I-Lys-plasmin(ogen) from Bacteria

^{125}I-Lys-plasminogen (approx. 100,000 cpm) was added to a 100 µl aliquot of a 10% w/v solution of strain 64/14 bacteria in a total volume of 400 µl VBS-gel and allowed to incubate at 37°C for 30 min. The bacteria were then pelleted by centrifugation (3000 x g, 10 min) and

washed three times with 2.0 ml VBS-gel. The bacterial pellets were resuspended in 300 μl of either VBS-gel containing 0.5% SDS; VBS-gel containing 0.1M EACA; or VBS-gel containing 0.5% SDS and 2.0% β-mercaptoethanol, to elute the ^{125}I-Lys-plasminogen from the bacteria. Following a 10 minute incubation at 37°C the bacteria were removed by centrifugation and the supernatant recovered. The eluted material was analyzed by electrophoresis on a 10%-SDS-PAGE-6M-Urea gel under reducing conditions. The gel was dried and the migration of labeled protein determined by autoradiography. Similar studies were also carried out in which the bacterial bound ^{125}I-Lys-plasminogen was treated with a 20 unit/ml concentration of urokinase for 20 minutes at 37°C in a total volume of 300 μl of VBS-gel prior to eluting the bound proteins. Following this plasminogen activation reaction the bacteria were centrifuged and washed twice with 2.0 mls VBS-gel. The residual bound ^{125}I-Lys-plasmin(ogen) was eluted and analyzed as described above.

Measurement of Functional Activity of Plasmin(ogen) in Bacterial-Free Supernatants

The following assay was used to measure binding of the various plasmin(ogen) as an alternative method to using radiolabeled tracers. In these studies, 2.0 μg of Glu-plasminogen, Lys-plasminogen, or Lys-plasmin was incubated with 100 μl of a heat killed 10% w/v suspension of the group A streptococcal strain 64/14 for 20 minutes at 37°C in a total reaction volume of 400 μl of VBS-gel. Following incubation, the bacteria were removed by centrifugation at 12,000 x g for 4 minutes in an Ependorf Microfuge and bacterial-free supernatants were obtained. Control tubes for each plasmin(ogen) species containing no bacteria were treated identically and all samples were run in duplicate. The bacterial free supernatants were recovered and enzymatic activity was measured as

follows. The bacterial free supernatants or the corresponding control samples were added to plastic cuvettes containing 10^6 IU streptokinase in a total volume of 900 μl of enzyme assay buffer (0.05 M Tris, 0.05 M NaCl, 0.1% PEG-8000, pH 7.4). For the Glu-and Lys-plasminogen preparations the reaction mixture was incubated at 37°C for 10 minutes to allow plasminogen-streptokinase complexes to form. For Lys-plasmin, a similar incubation with streptokinase was performed to allow for equivalent substrate turnover to that of zymogen-streptokinase complexes. Following incubation, H-D-Val-Leu-Lys-pNA (S-2251) was added to yield a final concentration of 300 μM. Tubes were allowed to incubate for precisely five minutes and then quenched with 100 μl of glacial acetic acid. The amount of substrate hydrolysis, which is directly proportional to the amount of plasmin enzyme present was then quantified by measuring the absorbance of the reaction mixture at 405 nm.

The enzymatic activity of the bacterial free supernatant was determined by comparison with the enzymatic activity of known standards. The percent of residual enzymatic plasmin(ogen) activity in the bacterial free supernatant was calculated by determining the fraction of total enzymatic activity in a control sample remaining in the supernatant following incubation with bacteria. Control tubes containing bacteria and substrate, and substrate in buffer were included. All assays were performed in duplicate.

Measurement of Functional Activity of Plasmin Associated with Bacteria

The plasmin activity associated with bacterial pellets was examined using the chromogenic substrate as described above. Following binding and centrifugation the pellets were washed 3 times with 1.0 ml of VBS-gel and resuspended in 400 μl of the enzyme assay buffer. S-2251 was added

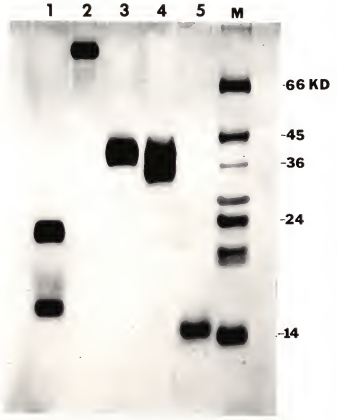
to yield a final concentration of 300 μM. The resuspended bacterial pellets were then incubated at 37°C for 20 minutes and quenched with 50 μl of glacial acetic acid. The bacteria were removed by centrifugation (12,000 x g for 4 minutes) and the optical density of the bacterial free supernatant was measured at 405 nm. Control tubes containing bacteria and substrate, and substrate in buffer were included. All assays were performed in duplicate.

Results

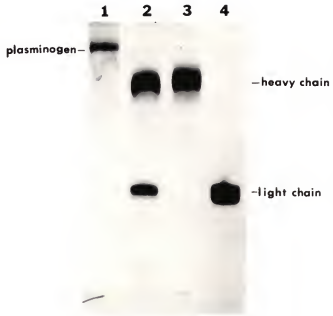
The experiments described in this chapter were designed to map the domains on the human plasmin molecule involved in the high affinity interaction with the group A streptococcal strain 64/14. For these studies a variety of defined plasminogen fragments as well as the heavy and light chains of plasmin were prepared as described in the Methods. The plasminogen fragments obtained were characterized on urea gels, see Figure 3-1. The homogeneous plasminogen fragments were used to compete with intact PPACK reacted ^{125}I-plasmin for receptor sites on the group A streptococcal strain 64/14 (Table 3-1). I have previously demonstrated that plasmin treated with PPACK, p-Nitrophenyl-p-guanidinobenzoate (pNpGB), or aprotinin, does not effect plasmin's binding reactivity to the group A streptococcal strain 64/14 as documented in Chapter Two. Enzymatic inhibition of the ^{125}I-Lys-plasmin and urokinase in the labeled tracer preparation was necessary to prevent the proteolytic conversion of Glu-plasminogen to Lys-plasminogen, or Lys-plasmin (Markus et al., 1978) by the labeled tracer mixture. In all the competitive inhibition experiments described in this study, a constant concentration of PPACK-^{125}I plasmin (1.0×10^{-10} M) and a range of concentrations of unlabeled

Figure 3-1. SDS-UREA-PAGE analysis of isolated plasmin(ogen) fragments. Panel A: Elastase digestion fragments of plasmin(ogen): Glu-plasminogen was digested with elastase and fragments purified as described in Materials and Methods. Panel A: Lane 1: Mini-PLG (4.0 μg); 2: Glu-PLG (4.0 μg); 3: Mini-PLA (4.0 μg); 4: LBS-I (4.0 μg); 5: LBS-II (4.0 μg); M: molecular weight standards. Panel B: Plasmin heavy (HC) and light (LC) chain preparations: Lys-plasmin was reduced and carboxymethylated as described in Materials and Methods. Lane 1: Glu-PLG (5.0 μg); 2: lys-PLA (5.0 μg); 3: HC (5.0 μg); 4: LC (5.0 μg). Proteins were electrophoresed under reducing conditions on a SDS-6 M urea-10%-polyacrylamide gel.

A


1
2
3
4
5
M
66 KD
45
36
24
14

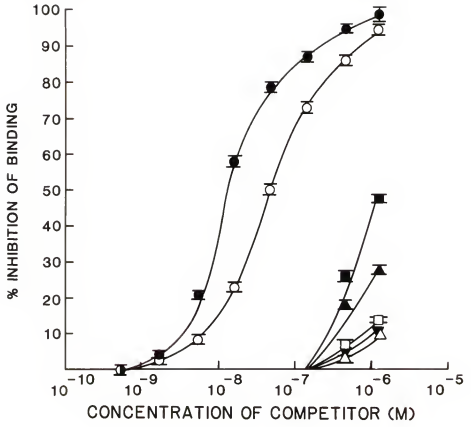
B


1
2
3
4
plasminogen
heavy chain
light chain

competitor molecules (10^{-6} M to 10^{-10} M) were mixed with a fixed concentration of bacteria. Following incubation and washing, the amount of radiolabeled plasmin bound to the bacterial pellet was determined. The quantity of radioactivity bound in the presence or absence of unlabeled competitor was compared and the degree of inhibition calculated (Figure 3-2). The results summarized in Table 3-1 show that unlabeled plasmin inhibits the binding of labeled plasmin efficiently, with 50% inhibition being observed in the presence of 1.2×10^{-8} M Lys-plasmin. Significant inhibition of radiolabeled plasmin binding was also observed when purified heavy chain was used as the competitor. Addition of any of the other plasminogen fragments including isolated lysine binding domains of the heavy chain (LBS I or LBS II) demonstrated no significant inhibitory effect (Figure 3-2). Similarly, mini-plasminogen, mini-plasmin, and isolated light chains demonstrated no significant inhibition of binding of radiolabeled lys-plasmin over the concentration range tested (10^{-6} M to 10^{-10} M) (Figure 3-2). Identical results were obtained in the inhibition assays involving mini-plasmin, mini-plasminogen, LBS I, LBS II, Lys-plasmin heavy chain, and plasmin light chain in the absence of protease inhibitors in the reaction mixture (data not shown).

Combining equimolar quantities of the elastase digested fragments of plasminogen or plasmin failed to restore any inhibitory potential. Furthermore, combination of isolated light chain and heavy chain demonstrated no synergistic effect in inhibitory capacity compared to the sum of the isolated fragments alone (data not shown). The inhibition curves for isolated heavy chain and intact plasmin (Figure 3-2) demonstrated that both preparations could inhibit binding of labeled Lys-plasmin by 100%. However, these curves differed in shape, indicating

Figure 3-2. Inhibition of PPACK reacted ^{125}I-Lys-plasmin binding to group A streptococcal plasmin receptor: A constant concentration of (1.0×10^{-10} M) PPACK reacted ^{125}I-Lys-plasmin and an increasing concentration of unlabeled competitor molecules (10^{-10} M to 10^{-6} M) were mixed with a fixed concentration of streptococcal strain 64/14. Following incubation and washing (see Materials and Methods), the amount of radiolabeled Lys-plasmin bound to the bacterial pellet was determined. The quantity of radioactivity bound in the presence of unlabeled competitor was compared to the radioactivity bound in the absence of inhibitor and the percent inhibition calculated. (● -Lys-plasmin; ○ -HC; ■ -LBS-I; □ -LBS-II; ▲ -LC; △ -Mini-PLG; ▼ -Mini-PLA).


% INHIBITION OF BINDING
100
90
80
70
60
50
40
30
20
10
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
CONCENTRATION OF COMPETITOR (M)

differences in the efficiency of inhibition. The isolated heavy chain was found to be less efficient an inhibitor than the intact plasmin molecule. These findings suggest that there is some component involved in the interaction of plasmin with the bacteria that is either not present on the heavy chain or is altered during the isolation procedure.

Two possibilities to account for these observations were considered. The first was that there are some sites on the heavy chain of the plasmin molecule that are modified when the molecule is purified, thereby changing its efficiency of interaction with the bacterial receptor. The second was that the plasmin light chain, while associated with the heavy chain, confers a different tertiary structure to the molecule than exists on either (or both) of the isolated chains. Such a change in conformation of the molecule might affect its interaction with the bacteria.

It has been established previously that a conformational change occurs when Glu-plasminogen is activated to Lys-plasmin, or when Glu-plasminogen is converted to Lys-plasminogen (Swenson and Thorsen, 1981; Markus et al., 1978; Thorsen, 1975). Lys-plasminogen is the zymogen form of plasminogen, lacking the 76 amino acid NH_2-terminus of the native protein (Markus et al., 1978). This modification results from the proteolytic activity of plasmin on Glu-plasminogen, which removes the 76 amino acid NH_2-terminus, resulting in a new NH_2-terminus lysine (for review, see Thorsen et al., 1981). This modification occurs without generation of protease activity. The conversion of Glu-plasminogen to Lys-plasmin or to Lys-plasminogen not only results in a marked conformational change of the protein but also causes an increase in the binding affinity of these molecules to fibrin (Thorsen, 1975), as well as

lowering the dissociation constant between these molecules and α_2-antiplasmin (Swenson and Thorsen, 1981; Wiman et al., 1979).

To examine the possible importance of the conformation of the plasmin(ogen) molecule for binding to bacteria, the ability of the conformationally altered form of plasminogen, Lys-plasminogen, to bind to the group A streptococcus, 64/14, was measured. The isolated protein was radiolabeled and examined by urea gel analysis for homogeneity. The labeled material demonstrated a single band on an autoradiograph (Figure 3-3, panel A, lane 2) at a position corresponding to that reported for the migration of Lys-plasminogen in this gel system (Swensen and Thorsen, 1981). This labeled form of plasminogen was found to bind to the bacteria (Figure 3-3, panel B, lane 2). Similarly, Glu-plasmin generated from Glu-plasminogen in the presence of aprotinin, was also capable of binding to the bacteria (Figure 3-3, panel B, lane 3). The relative efficiency of unlabeled Glu-plasminogen, Lys-plasminogen and Lys-plasmin to compete with labeled plasmin for binding sites on the group A streptococcus 64/14 was tested. Different concentrations of each of these molecules were mixed with a fixed concentration (1.0×10^{-10} M), of PPACK reacted ^{125}I-Lys-plasmin and the extent of inhibition of binding of radiolabel was measured, as described previously. The results of this experiment shown in Figure 3-4 indicate that the inhibition achieved with Lys-plasminogen and Lys-plasmin were identical. These results indicate that the receptor for these ligands are the same, and that the affinity for each protein is equivalent.

The possibility that the results presented in Figure 3-4 could be accounted for by the conversion of Lys-plasminogen to Lys-plasmin during the reaction was considered. The next series of experiments were

Figure 3-3. Binding of ^{125}I labeled Glu- and Lys-plasmin(ogens): Glu- and Lys-plasmin(ogen) were generated as described in Materials and Methods. The labeled tracers were then used in direct binding assays with a fixed concentration of the streptococcal strain 64/14. Panel A is an autoradiograph demonstrating the analysis of each reduced ^{125}I-labeled sample on a SDS-6 M-Urea 12%-polyacrylamide gel by autoradiograph to verify their molecular form. (Glu-H: Glu-heavy chain; Lys-H: Lys-heavy chain; L: light chain). Panel B illustrates the percent of offered cpm bound to bacterial pellets. (Lane 1: Glu-PLG; 2: Lys-PLG; 3: Glu-PLA; 4: Lys-PLA).

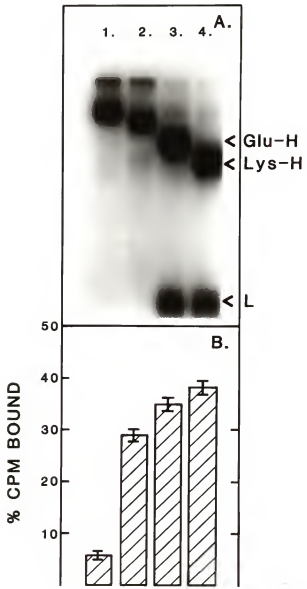
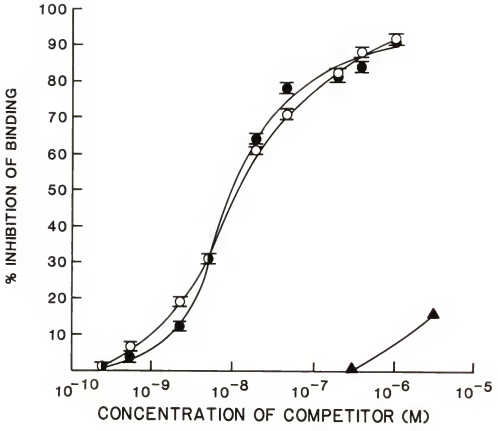

A.
1.
2.
3.
4.
Glu-H
Lys-H
L
B.
% CPM BOUND
50
40
30
20
10

Figure 3-4. Inhibition of PPACK reacted ^{125}I-lys-plasmin binding to group A plasmin receptor: A constant concentration of (1×10^{-10} M) PPACK reacted ^{125}-Lys-plasmin and an increasing concentration range of Lys-PLG, Glu-PLG or Lys-PLA (10^{-10} M to 10^{-6} M) were mixed with a fixed concentration of the streptococcal strain 64/14. Following incubation and washing (see Materials and Methods), the amount of radiolabeled Lys-plasmin bound to the bacterial pellet was determined. The quantity of radioactivity bound in the presence of unlabeled competitor was compared to the radioactivity bound in the absence of inhibitor and the percent inhibition calculated. (○-Lys-PLA; ● -Lys-PLG; ▲ - Glu-PLG).


% INHIBITION OF BINDING
100
90
80
70
60
50
40
30
20
10
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
CONCENTRATION OF COMPETITOR (M)

designed to determine whether Lys-plasminogen binds to the bacteria without first being activated. These experiments were carried out by monitoring the distribution of Lys-plasminogen, Glu-plasminogen, or Lys-plasmin, in the fluid phase and associated with the bacteria, following incubation of the protein with the bacteria. Unlabeled Glu-plasminogen, Lys-plasminogen, or Lys-plasmin was added to a fixed concentration of the group A streptococci 64/14 and incubated for 30 minutes at 37°C. Following this incubation period, the bacteria were pelleted by centrifugation and the supernatants were recovered and monitored for enzymatic activity either directly for Lys-plasmin, or following activation with excess streptokinase for the sample containing Glu-plasminogen or Lys-plasminogen, as described in the Methods. Following incubation with bacteria, and removal of the bacteria by centrifugation, there was no significant Lys-plasmin activity detectable in the bacterial free supernatant (Figure 3-5). By contrast over 98% of the enzymatic potential of Glu-plasminogen was detected in the supernatant, while in similar experiments using Lys-plasminogen less than 10% of the enzymatic potential was measured following activation with streptokinase (Figure 3-6). Because of differences in the efficiency of detection of plasmin activity in the fluid phase compared to its activity when bound to bacteria it is not possible to quantitate accurately the exact percentage of plasmin activity that is bound to bacteria. However, I have demonstrated previously that once associated with bacteria, the plasmin retains its ability to cleave synthetic chromogenic substrates like H-D-val-leu-lys-pNA (S-2251), as documented in Chapter Two. Consequently, the washed pellets from the absorption reaction were incubated with this synthetic substrate. The results presented in Table 3-2 demonstrate

Figure 3-5. Binding of Lys-plasmin(s), derived from Glu-plasminogen and Lys-plasminogen, to the group A streptococcal strain 64/14 as measured by residual activity in the bacterial free supernatant: (○ -urokinase activated Glu-plasminogen alone; ● -urokinase activated Glu-plasminogen + bacteria; □ -urokinase activated Lys-plasminogen alone; ■ -urokinase activated Lys-plasminogen + bacteria). For precise experimental details see Materials and Methods.

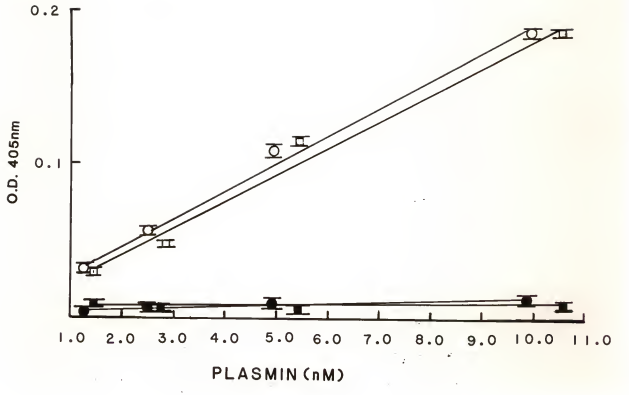
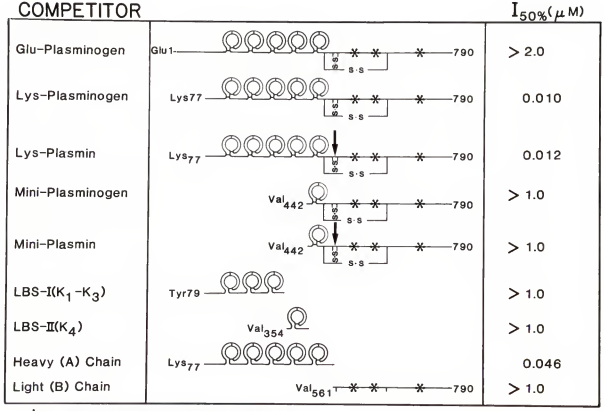

0.2
0.1
O.D. 405nm
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
PLASMIN (nM)

Table 3-1.

Summary of inhibition experiments of PPACK reacted ^{125}I-Lys-plasmin binding to the group A streptococcal strain 64/14 are shown with a schematic depiction of the portion of the native molecule they represent.**



↓ Activation cleavage site (arginine$_{560}$–valine$_{561}$).

* Plasmin active site residues (histidine$_{602}$; aspartic acid$_{645}$; serine$_{740}$) from left to right.

**Structure and NH_2-amino-terminal residue data were obtained from the work of Sottrup-Jensen et al., (1978). Inhibition is expressed as 50% inhibitory values in (μM) with Lys-plasmin as the standard (see Materials and Methods).

Table 3-2.

Measurement of plasmin(ogen) associated with bacterial pellets.*

Bacteria Pre-Incubated With:	Absorbance at 405 nm Following a 20 Minute Incubation at 37°C with H-D-Val-Leu-Lys-Paranitroanilide
Buffer	0.023 ± 0.002
Glu-plasminogen	0.026 ± 0.002
Lys-plasminogen	0.095 ± 0.004
Lys-plasmin[1]	0.630 ± 0.001
Lys-plasmin[2]	0.637 ± 0.002

*Two μg of the indicated enzyme or zymogen was incubated with a fixed dilution of the streptococcal strain 64/14. Following incubation the bacteria were pelleted by centrifugation, washed, resuspended in 400 μl VBS-gelatin, and assayed for enzymatic activity by hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-paranitroanilide (see Materials and Methods). The spontaneous cleavage of the substrate under the experimental conditions in the presence of bacteria alone was an absorbance (405 nm) of 0.024 ± 0.002.

1. Urokinase activated Glu-plasminogen

2. Urokinase activated Lys-plasminogen

Figure 3-6. Binding of Glu- and Lys- plasminogen to the group A streptococcal strain 64/14 as measured by residual activatable zymogen in the bacterial free supernatant: (● - Glu-plasminogen + bacteria; ○-Glu-plasminogen alone bacteria; ■-Lys-plasminogen + bacteria; □-Lys-plasminogen alone). For precise experimental details see Materials and Methods.

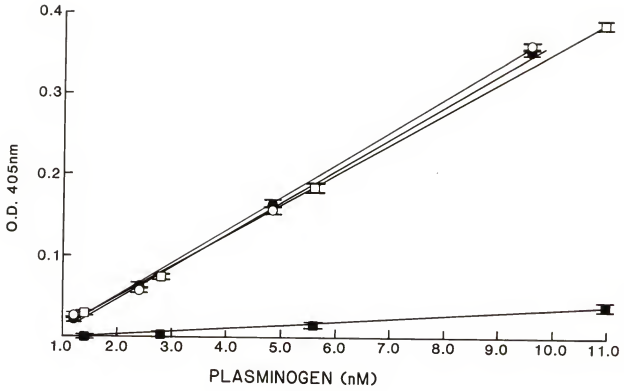
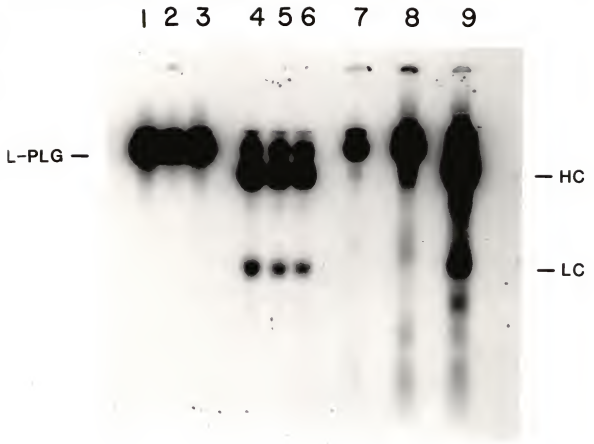

0.4
0.3
0.2
0.1
O.D. 405nm
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
PLASMINOGEN (nM)

Figure 3-7. Characterization of ^{125}I-Lys-plasmin(ogen) species eluted from bacteria: Eluted labeled proteins were analyzed by electrophoresis on a 10%-SDS-PAGE-6M urea gel under reduced conditions. Lanes 1, 2, and 3 contain labeled proteins from bacteria pre-incubated with ^{125}I-Lys-plasminogen and eluted by 0.5% SDS; 0.1 M EACA; or 0.5% SDS containing 2.0% ß-mercaptoethanol respectively. Lanes 4, 5, and 6 are identical to Lanes 1, 2, and 3 with the exception that the bound labeled proteins were pre-incubated with urokinase prior to elution. Lane 7 contains ^{125}I-Lys-plasminogen incubated at 37°C without bacteria for the period of the experiments and Lanes 8 and 9 contain ^{125}I-Lys-plasminogen and ^{125}-Lys-plasmin respectively. For precise experimental details see Methods.


1 2 3 4 5 6 7 8 9
L-PLG —
— HC
— LC

that the bacteria incubated with Lys-plasminogen exhibited only a low level of enzymatic activity (approximately 15% of that observed in the samples pre-incubated with Lys-plasmin). The bacterial free supernatant of the sample incubated with Lys-plasminogen demonstrated <10% of the zymogen remained in the supernatant as detected by enzymatic activity following activation with streptokinase (Figure 3-6). Taken together these results indicate that the Lys-plasminogen was removed from the fluid phase without prior or concomitant activation to Lys-plasmin. Furthermore, when the bacterial bound ^{125}I-Lys-plasminogen was eluted from the bacteria and examined by polyacrylamide gel electrophoresis under reducing conditions, a single protein band was observed on the autoradiograph corresponding to the enzymatically inactive modified zymogen form of the protein, Lys plasminogen (Figure 3-7). All of these studies demonstrate that Lys-plasminogen can bind to bacteria without first being converted to Lys-plasmin. This would indicate that the intact native plasminogen molecule (Glu-plasminogen) does not express structures that are recognized by the bacterial plasmin receptor. However, following a conformational change achieved by either conversion to the Lys-plasminogen form of the zymogen or by activation to plasmin, structures are formed or exposed on the molecule that facilitate interaction with the bacteria.

Discussion

Plasmin is the key component of the mammalian fibrinolytic enzyme system which is responsible for fibrin degradation and intravascular blood clot lysis. Active plasmin, which cleaves fibrin, is derived from the circulating zymogen precursor Glu-plasminogen. Glu-plasminogen is a

single chain glycosylated protein containing 790 amino acids in known sequence with a molecular weight of approximately 92,000 daltons (Thorsen et al., 1981; Wiman, 1973, 1977). The generation of plasmin from plasminogen is accomplished by proteins known as plasminogen activators. This conversion is brought about by cleavage of a single arginine (560)-valine (561) peptide bond which creates, through conformation changes, a two chain active plasmin molecule held together by disulfide linkages (Astrup, 1978). The light chain of plasmin has a molecular weight of approximately 25,000 daltons and contains the serine protease active site (Robbins and Summaria, 1970; Wiman, 1977). The heavy chain of plasmin has a molecular weight of approximately 63,000 daltons (Robbins and Summaria, 1970) and contains 5 homologous triple loop structures known as kringles (Sottrup-Jensen et al., 1978). An additional conformationally distinct form of plasminogen can be generated when Glu-plasminogen is exposed to plasmin. This removes a 76 amino acid peptide from the NH_2-terminus, thereby generating Lys-plasminogen (Swenson and Thorsen, 1981).

The plasmin(ogen) molecule contains several characteristic 'lysine-binding sites', one with high affinity for the lysine analogue EACA (dissociation constant of 9.0 μM), and four or five with low affinity (dissociation constant of 5 mM) (Markus et al., 1978a, 1978b). The high affinity site has been mapped to the kringle 1 region, and one of the lower affinity sites has been mapped to the kringle 4 region of the plasmin(ogen) molecule (Lerch et al., 1980). These structures are known to participate in the binding of plasmin(ogen) to α_2-antiplasmin (α_2-AP) (Wiman, 1981) and to fibrin (Swenson and Thorsen, 1981; Wiman et al., 1979) respectively. It is known that binding of lysine and lysine analogs to plasmin(ogen)'s lysine binding sites induces conformational

changes in the molecule (Violand et al., 1975). I have shown previously that lysine or α_2-AP inhibit the binding of plasmin to the group A streptococcal receptor, as documented in Chapter Two, indicating the possible involvement of the high affinity lysine-binding site in the plasmin-bacterial receptor interaction. A comparison of my findings with studies of the interaction of plasminogen with other naturally occurring plasminogen binding proteins reveals a number of interesting similarities and contrasts. Specific binding to the group A streptococcus, 64/14, was demonstrated with plasmin's heavy chain. However, the isolated heavy chain alone was not as efficient a competitor as intact Lys-plasmin, as evidenced by the non-superimposible nature of the heavy chain and Lys-plasmin inhibition curves (Figure 3-2). It should be noted that 100% inhibition of binding of Lys-plasmin could be achieved by addition of high concentrations of heavy chain, but none of the kringle containing fragments (Lysine-binding domains) alone or in combination had any significant inhibitory effects at similar molar concentrations. This finding stresses the importance of the conformation of the entire heavy chain for binding to bacteria. The bacterial binding of plasmin therefore differs from the kind of interaction seen with α_2-AP, as well as with fibrin and fibrinogen, to which plasmin as well as plasminogen, LBS-I, LBS-II, and mini-plasmin(ogen) are known to interact (Swenson and Thorsen, 1981; Thorsen et al., 1981; Wiman et al., 1979).

Consistent with my initial observations, documented in Chapter Two, there is no significant binding of the native zymogen, Glu-plasminogen, while the conformationally altered form of the zymogen, Lys-plasminogen, was found to bind specifically to bacteria (Figures 3-3,3-4, and 3-5).

This form of the zymogen molecule is known to be conformationally distinct from Glu-plasminogen and is similar in conformation to Lys-plasmin (Violand et al., 1975). The binding of Lys-plasminogen to the group A streptococcal receptor is therefore dependent on a specific conformation, most probably of the heavy chain.

Interaction of both plasmin and plasminogen with thrombospondin (an adhesive glycoprotein) has been demonstrated to occur via interaction with the heavy chain of the plasmin(ogen) molecule (Silverstein et al., 1984; Walz et al., 1987). However, efficient binding of thrombospondin with any elastase digestion fragment of plasminogen has not been observed (Walz et al., 1987). I have observed a similar pattern for the interaction of plasmin(ogen) with its bacterial receptor. However, unlike the interaction of thrombospondin with plasmin, the bacterial binding properties were reversible by addition of lysine or lysine analogs (Broeseker et al., 1988).

Histidine-rich glycoprotein (HRGP), an α_2-glycoprotein in human plasma, has been reported to compete with α_2-AP for the high-affinity lysine-binding site in plasmin (Haupt and Heinburger, 1972; Lijnen et al., 1980). In addition, HRGP also reduces the binding of plasminogen to fibrin by complex formation with the low-affinity lysine binding sites (Lijnen et al., 1980). Furthermore, the characteristic interaction of Glu-plasminogen, Lys-plasminogen, or plasmin and their fragments with fibrin or fibrinogen involves the heavy chain lysine-binding sites (Cenderholm-Williams, 1977). This is distinct from the profile of reactivity for the interaction of these proteins with the group A streptococcus (Table 3-1). It can be seen that Glu-plasminogen shows no reactivity with these bacteria, nor is there any significant reactivity with the isolated lysine-binding fragments LBS I or LBS II.

Of particular relevance to this study is the interaction of plasmin(ogen) with the well characterized streptococcal plasminogen activator streptokinase isolated from group C streptococci (Christensen, 1945; Tillet and Garner, 1933). This secreted streptococcal protein is known to bind rapidly to Glu-plasminogen, Lys-plasminogen, and Lys-plasmin (rate constant 5.4 x 10^7 $M^{-1}S^{-1}$) forming a 1:1 stoichiometric complex with an estimated dissociation constant of 5 x 10^{-11} M (Cenderholm-Williams et al., 1979). This interaction occurs via an interaction with the light chain (Summaria and Robbins, 1976). The interaction with the group A streptococcal plasmin receptor is distinct from group C streptokinase in that it does not recognize the Glu-plasminogen molecule, and demonstrates no significant reactivity with the isolated light chain of plasmin. Furthermore, the plasmin(ogen)-streptokinase complex cannot be dissociated by lysine or lysine analogs (Von-Mering et al., 1988), while the interaction of plasmin with a group A streptococcus is completely reversible by lysine or lysine analogs (Broeseker et al., 1988).

Taken together, these results indicate that the group A streptococcal plasmin receptor binds in a unique manner to both plasmin and Lys-plasminogen. The predominant interaction is via determinants present on the intact heavy chain. These structures are present in their optimal binding configuration on the intact plasmin molecule and on the modified zymogen, Lys-plasminogen. The studies presented here suggest that the lysine binding sites themselves are not involved in direct interaction of plasmin with the bacteria (Figure 3-2). The observations that plasmin bound to bacteria retains its enzymatic activity for both small synthetic substrates and for fibrin, Chapter Two, are consistent with the observations that the light chain is not involved in binding.

The failure of α_2-AP to regulate the bound enzyme suggests that the required interaction between α_2-AP and plasmin is directly or indirectly inhibited. This may occur because one of the recognition sites for α_2-AP in the kringle 1 region of plasmin's heavy chain may not be accessible when plasmin is bound to a streptococcus.

The characteristics of the interaction of human plasmin with the group A streptococcus, 64/14, described in this study indicate that the bacteria can capture a potent protease activity that cannot be regulated by the primary physiological inhibitor of plasmin, α_2-AP. This group A streptococci also secretes a plasminogen activator and consequently, in the presence of plasminogen, the bacteria has the potential to both generate plasmin and bind the active enzyme to its surface (DesJardin et al., 1988). The importance of this selective receptor to the infectious disease process of receptor positive bacteria remains to be established.

The purpose of the series of studies described in the next chapter was to isolate and characterize the plasmin binding receptor from the strain 64/14 streptococcus.

CHAPTER FOUR
ISOLATION AND PURIFICATION OF A FUNCTIONALLY ACTIVE
GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN PLASMIN

Introduction

The studies presented thus far have documented the existence of a cell surface receptor for human plasmin on group A streptococcal strain 64/14. In addition to this plasmin binding activity, certain group A streptococci have long been known to secrete the plasmin(ogen) binding protein streptokinase, (M_r approx. 48,000 daltons), a non-enzymatic plasminogen activator. This protein, described by Tillet and Garner (1933), non-covalently associates with both plasminogen and plasmin, and was originally identified by virtue of its ability to generate fibrinolytic activity. Streptokinase binds rapidly to the native zymogen Glu-plasminogen (rate content 5.4 x 10^7 $M^{-1}S^{-1}$) forming, a 1:1 stoichiometric complex with an estimated dissociation constant of 5 x 10^{-11} M (Cederholm-Williams *et al.*, 1979). The formation of a complex between streptokinase and plasminogen generates an enzymatic moiety capable of plasminogen activator activity, a property neither protein possesses alone.

The properties of the bacterial plasmin receptor reported thus far are markedly different from streptokinase. While the bacterial plasmin receptor binds preferentially to domains in the heavy chain of the plasmin molecule (see Chapter Three), streptokinase binds to plasmin's light chain (Summaria and Robbins, 1976). Furthermore, streptokinase

binds to both plasmin and the native zymogen Glu-plasminogen, while the surface associated plasmin receptor shows no significant reactivity for the native zymogen. Despite these clear functional differences, the expression of two proteins by the same bacteria that bind to the key human fibrinolytic protein plasmin with such selectivity raises the possibility that they may be in some way related or derived from a common precursor. Furthermore, the majority of information on the properties of streptokinase have been derived from studies of the plasminogen activator molecule isolated from group C streptococcal strains and evidence for differences in antigenicity and hence possibly function have been reported between streptokinase proteins isolated from group A and group C streptococcal isolates (Dillon and Wannamaker, 1965; Weinstein, 1953). The purpose of the studies presented in this chapter were to isolate a functionally active receptor for human plasmin from strain 64/14 and to compare it with the streptokinase protein that is produced by the same organism.

Materials and Methods

Enzymes, Inhibitors and other Reagents

Urokinase and porcine elastase (type IV) were obtained from Sigma Chemical Co., St. Louis., MO. Aprotinin was obtained as Trasylol from Mobay Pharmaceuticals, New York, NY. D-Val-Phe-Lys-chloromethyl ketone (VPLCK), and Phe-Pro-Arg-chloromethylketone (PPACK) were obtained from Calbiochem-Behring, San Diego, CA. Human Lys-plasminogen was obtained from American Diagnostica Inc., Greenwich, CT. H-D-Val-Leu-Lys-paranitroanilide (S-2251) was obtained from Helena Laboratories, Beaumont, TX. Purified group C streptokinase was a gift from Kabivitrum, A.B., Stockholm, Sweden.

Bacteria

The Lancefield β hemolytic streptococcal strain 64/14 was grown as a stationary culture at 37°C, in one to two liter batches of a chemically defined media for streptococci described by Van de Rijn and Kessler (1980), containing 0.1% phenol red. The pH of the cultures were maintained at a pH greater than 7.0, as monitored by the indicator dye. For certain experiments, where noted, bacteria were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) Approximately 2.0 to 4.0 g (wet weight) of bacteria could be recovered per liter of media following a 24 to 36 hour incubation at 37°C. Bacteria were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.02% sodium azide. The bacteria were heat killed at 80°C for 15 min. The suspension was centrifuged and the pellet washed twice with PBS containing 0.02% sodium azide. Aliquots could be stored at -20°C, or used immediately for extraction purposes.

Radioiodination of Proteins

Human plasminogen was iodinated by the chloramine T method using Iodobeads (Pierce Chem. Co., Rockford, IL) as described by Markwell (1982). The labeled proteins were separated from free iodine by passage over a G25 column (PD-10 Pharmacia) and collected in 0.15 M Veronal buffered saline pH 7.35 containing 0.001 M Mg^{++}, 0.00015 M Ca^{++} and 0.1% gelatin (VBS-gel). The labeled proteins were stored in aliquots containing 0.02% sodium azide at -20°C. Labeled aliquots were used once and discarded.

Generation of Plasmin

Plasmin was generated from either radiolabeled or unlabeled plasminogen by reaction with urokinase. Three μl of urokinase (Sigma 20

u/ml) was added to a 400 μl solution of 1 μM plasminogen containing 0.04 M lysine. The mixture was incubated at 37°C for 45 minutes unless stated otherwise. The efficiency of plasmin generation was followed by measuring the conversion of the single chain plasminogen molecule (M_r= 90,000 daltons) into heavy chains (M_r=60,000 daltons) and light chains (M_r=25,000 daltons) as determined by the migration of radiolabeled proteins, following reduction, on 10% SDS-polyacrylamide gels. The migration of labeled proteins was determined by autoradiographic exposure of dried gels to Kodak XAR 5 film with intensifying screens at -70°C for 15-20 hours.

Direct Binding Assay of Radiolabeled Proteins

The ability of radiolabeled plasmin(ogen) to bind to the group A streptococcus 64/14 was measured as described previously by Lottenberg et al., (1987). A fixed number of bacteria were incubated with labeled proteins (approximately 30,000 cpm per tube) in a total volume of 400 μl of VBS-gel for 30 min at 37°C. The bacteria were pelleted by centrifugation at 1000 x g for 10 min and the pellets washed twice with 2.0 ml of VBS-gel. The radioactivity associated with the bacteria was determined in a Beckman 5500 Auto gamma counter (Beckman Instruments, Inc., Fullerton, CA). Non-specific background binding was determined in replicate tubes which contained no bacteria. All estimates were performed in duplicate.

Dot-blotting Procedure for the Identification of Plasmin Receptor Activity

This assay was carried out with a Bio-Rad bio-dot microfiltration apparatus using a modification of the Bio-Rad procedure. A piece of nitrocellulose pre-equilibrated in PBS-azide for a minimum of 10 minutes was fitted into the apparatus. The wells were loaded with 100 μl

aliquots of PBS-azide and vacuum drained. Extraction samples, chromatography fractions or standards were loaded into wells in 50-200 μl aliquots. Commercially available group C streptokinase (Kabikinase) was used as a positive control in each assay. All wells were washed twice with 200 μl aliquots of PBS-azide and vacuum drained, all samples were assayed in duplicate.

Blots were removed from the apparatus and remaining sites on the nitrocellulose were blocked by washing a total of four times in 200-250 ml of 5.0 mM sodium diethylbarbiturate, 0.14 M NaCl, 0.5% gelatin, 0.15% Tween 20, 0.004% NaN_3 pH 7.35 (blotting wash buffer I) for 15 minutes per wash. At this point, blots could be probed as described below or stored in the fourth wash overnight at 4-8°C. If the latter procedure was followed, blots were washed a fifth time after cold storage in 200-250 ml blotting wash buffer I for 30 minutes. Results from the two variations did not differ.

The individual blots were then probed for 3-4 hours at room temperature while rotating in 10 ml aliquots of the following probing solution: blotting wash buffer I containing 2.0 mM PMSF and ^{125}I-labeled plasmin at 2 x 10^5-3 x 10^5 cpm/ml. The probed blots were then washed four times in 200-250 ml of 0.01 M EDTA, 0.5 M NaCl 0.25% gelatin, 0.15% Tween 20, 0.004% NaN_3 for 15 minutes per wash. All washing and probing steps were carried out at ambient temperature. The probed, washed blots were air dried.

Autoradiographs were prepared by exposing the nitrocellulose blots to Kodak XAR-5 film with an intensifying screen for 15-24 hours at -70°C followed by automated film developing.

Polyacrylamide Gel Electrophoresis and Protein Blotting

Electrophoresis was carried out as described by Laemmli (1970). Polyacrylamide separating gels were 10% and contained 0.1% sodium dodecylsulfate (SDS), 0.375 M Tris at pH 8.8. Stacking gels were 4% and contained 0.1% SDS and 0.125 M Tris at pH 6.8. Electrode buffer was 0.024 M Tris, 0.192 M glycine, 0.1% SDS at pH 8.3 Samples were diluted 1:2 with sample buffer containing 0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue and heated at 80-90°C for 3 minutes. Gels were run at 45 volts constant voltage for approximately 15-18 hours. Slab gels were used in the Bio-Rad Protean II system (BioRad, Richmond, CA). Molecular weight markers were run on all gels. Gels intended for Western blot transfer contained pre-stained markers (Sigma) applied as a mixture which included: triosephosphate isomerase (26,600), lactic dehydrogenase (36,500), fumarase (48,500), pyruvate kinase (58,000), fructase-6-phosphate kinase (84,000), β-galactosidase (116,000), and α_2-macroglobulin (180,000). After electrophoresis, gels intended for Western blotting were equilibrated in 25 mM Tris, 0.2 M glycine pH 8.0 containing 20% v/v methanol (electroblot buffer) for 25 minutes. Protein blotting, from SDS-PAGE gels, was performed using the 'Trans-Blot SD Semi-Dry' electrophoretic transfer cell (Bio Rad, Richmond, CA). Nitrocellulose transfer medium, also equilibrated in electroblot buffer, was sandwiched between the gel and two sheets of Whatman 3 mm paper. The gel was also backed with two sheets of 3 mm paper. For probing with plasmin, the Western blots were washed, probed and autoradiographed according to the procedure described above for dot-blotting. For probing with rabbit, anti-plasmin receptor antibody, blots were probed with blotting wash buffer I containing 4.3 μg

IgG per ml of probing solution (approximately a 1:3000 dilution of antisera) for three hours, washed twice for 20 min with 300 mls of blotting wash buffer I, and probed with ^{125}I-Protein G at 2 x 10^5-3 x 10^5 cpm/ml, the probed blots were then washed four times in 200-300 mls of 0.01 M EDTA, 1.0 M NaCl 0.25% gelatin, 0.15% Tween 20 for 15 minutes per wash. All washing and probing steps were carried out at ambient temperature. The probed, washed blots were air dried.

Autoradiographs were prepared by exposing the nitrocellulose blots to Kodak XAR-5 film with an intensifying screen for 15-24 hours at -70°C followed by automated film developing.

Molecular weight determinations on Western blots were made possible by the transfer of the prestained molecular weight markers.

Gels to be used for protein identification were either stained with silver according to the procedure described by Merril et al., (1981), or with Coomassie brilliant blue R-250 as follows: (0.25% w/v in 40% ethanol and 10% acetic acid) for 1 hour, and destained by soaking in several changes of 10% ethanol and 10% acetic acid containing a small quantity of DE 52 (Whatman, England) as a dye adsorbent.

Lancefield Hot Acid/Hot Alkaline Extractions

These extractions were carried out as described by Lancefield (1928). Approximately 10 ml aliquots of 10% (wet weight/volume) 64/14 in PBS-azide were adjusted to pH 2 or 10 using 0.5% M HCl or 0.5 M NaOH respectively. The suspensions were boiled for 10 minutes and the pH was neutralized in each sample using either 0.5 M NaOH or 0.5 M HCl. PMSF was added to a concentration of 2.0 mM. The samples were centrifuged at approximately 10,000 x g for 10 minutes. The supernatants were passed through a 0.22 μm filter to remove any residual cells. The cell-free

supernatants were then dialyzed at 4°C into 20 mM Tris-HCl, 0.15 M NaCl pH 7.4 containing 1.0 mM iodoacetic acid, 1.0 mM benzamidine HCl,and were stored at -70°C.

Time Course Trypsin Digestion

The bacterial pellet from approximately 11 ml of 10% (w/v) 64/14 in PBS-azide was collected by centrifugation at 10,000 x g for 10 minutes. The pellet was washed with 10 ml of 0.05 M KH_2PO_4, 0.005 M EDTA, 0.02% NaN_3 pH 6.1, centrifuged as before and resuspended to 10% (w/v) in that buffer. These salt and buffer conditions are not optimal for trypsin activity and facilitate the extraction of surface proteins without concomitant proteolysis of the solubilized material. Pancreatic DNAse I (Sigma) was added to approximately 6.0 ml of this suspension to a final concentration of 4 μg/ml. The sample was vortexed and warmed to 37°C. Bovine pancreatic trypsin (Type I, Sigma) was then added to a final concentration of 20 μg/ml and the sample was mixed. A 1.0 ml aliquot was immediately removed and mixed with a concentrated solution of benzamidine HCl. The final concentration of benzamidine HCl in the reaction mixture was 100 mM, well in excess of what was required to completely inhibit the activity of the trypsin present in the reaction mixture. This sample, was mixed and placed on ice, and was designated the zero time of the experiment. At 5, 10, 30 and 60 minutes 1.0 ml aliquots were removed from the reaction mixture, and were treated in an identical manner. A control digestion was prepared by incubating a 1.0 ml aliquot of 10% bacterial suspension containing 4 μg/ml DNAse I at 37°C for 60 minutes followed by the addition of benzamidine HCl to 100 mM final concentration. All samples were centrifuged at approximately 10,000 x g for 10 minutes. Supernatants were collected and stored at -70°C. Prior

to testing each control and experimental supernatant was centrifuged for 5 minutes at 10,000 x g to remove any particulate material. A control sample treated in an identical fashion with the exception that no trypsin was added was included at the 60 minute incubation time to determine the degree of non-specific release of proteins from the bacteria. In addition a control from which bacteria were omitted was included in each assay.

Non-ionic Detergent/Osmotic Shock/Lysozyme Extraction

This extraction was a modification of the procedure described by Scopes (1982). Approximately 1.0 g wet weight of 64/14 was combined with 2.5 ml of glycerol and 0.1 ml of 10% (v/v) Triton X-100 in 20 mM KH_2PO_4, 1.0 mM EDTA 0.02% NaN_3 pH 7.6. The cells were dispersed by vortexing and the sample was placed at 37°C for 30 minutes. The suspension was vortexed a number of times while incubating. Following incubation, the mixture was adjusted to 20 ml with lysozyme buffer. Lysozyme (Sigma) was added to 200 μg/ml and DNAse I to 10 μg/ml. The sample was vortexed and returned to 37°C for 30 minutes with frequent vortexing. After incubation, PMSF was added to 2.0 mM final concentration. The supernatant was collected and treated as described for the Lancefield extractions. As enzyme control to be tested with the extract in the screening assay for plasmin receptor activity contained 200 μg/ml lysozyme and 10 μg/ml DNAse I in 20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1.0 mM iodoacetic acid, 1.0 mM benzamidine HCl and .02% NaN_3.

Acetone/Detergent Extraction

This procedure was a modification of the extraction described by Bhaduri et al., (1983). approximately 1.0 g wet weight of 64/14 was suspended in 10 ml of ice cold acetone (Fisher Certified A.C.S. grade),

allowed to stand on ice for 5 minutes and then collected by centrifugation at 10,000 x g for 10 minutes. Residual acetone was evaporated under a stream of air. The pellet was resuspended with vortexing in 25 ml of 1.0% (v/v) Triton X-100 in PBS-azide and incubated at room temperature for 5 minutes. An additional 2.5 ml of PBS-azide was added. The supernatants were collected and treated as described for the Lancefield extracts.

Mutanolysin Extraction

This procedure is a modification of the method described by Yarnall et al., (1986). approximately 0.9 g wet weight of 64/14 was suspended in 5.0 ml of 20 mM KH_2PO_4, 1.0 mM EDTA, .02% NaN_3 pH 7.0 containing 2.0 mM PMSF, 10 μg/ml DNAse I and 50 μg/ml mutanolysin. The mutanolysin was purified from a commercial product (Sigma) according to the method described by Siegal et al., (1981). The suspension was vortexed and placed at 37°C for 4 hours with periodic vortexing. Supernatants were collected and treated as described above for Lancefield extractions. An enzyme control for use in the plasmin receptor assay contained 10 μg/ml DNase I, 2.0 mM PMSF, and 50 μg/ml mutanolysin in 20 mM Tris-HCl, 0.15 M NaCl, 1.0 mM iodoacetic acid, 1.0 mM benzamidine HCl, 0.02% NaN_3 pH 7.4.

Preparation of Immobilized Human Plasmin Affinity Column

Human plasminogen at a concentration of approximately 5.2×10^{-5} M was activated to plasmin by incubating the sample in the presence of an approximately 62 fold lower molar concentration of urokinase (Abbott). The reaction volume was 10 ml and the primary buffer was 0.05 M Tris, 0.15 M NaCl pH 7.4 containing 40 mM lysine. Conversion was carried out with constant agitation for one hour at 37°C. A 50 μl aliquot was removed and the remainder flash frozen and stored at -70°C. The sample

taken was analyzed by SDS-PAGE under reduced conditions for the conversion of the single chain plasminogen molecule to the two chain plasmin form. Once it was established that the plasminogen was fully activated, the bulk preparation was reacted with a 5 fold molar excess of D-valyl-L-phenylalanyl-L-lysine chloromethyl ketone (VPLCK) (Calbiochem), an irreversible inhibitor of the enzyme activity of plasmin. This enzyme inactivation was carried out at ambient temperature with constant rotation. The enzymatically inactive plasmin was then concentrated by ammonium sulfate precipitation (4.0 g / 10 ml), and dialyzed at 4°C against 0.1 M MOPS buffer, pH 7.3, containing 0.02% sodium azide. The dialyzed inactive plasmin was then chromatographed on Superose 6 (Pharmacia) in 0.1 M MOPS buffer, pH 7.3.

The activated affinity chromatography support Affi-Prep 10 (Bio Rad) was selected as the matrix for immobilizing the chlormethyl ketone blocked plasmin. This matrix couples in aqueous buffers by means of an N-hydroxysuccinimide ester on the end of a 10 carbon space arm to primary amino groups in the ligand. The ligand is linked by amide bonds to the terminal carboxyl groups of the Affi-Prep 10 spacer arm. The buffer used in the coupling reaction was 0.1 M MOPS buffer, pH 7.3. Approximately 50 mg of inactivated plasmin in 18 ml of coupling buffer was incubated with 6.0 ml of washed Affi-Prep 10. The reaction was carried out at 4°C for 15 hours with rotation. Following ligand coupling, 100 μl of 1.0 M ethanolamine HCl pH 8 was added to the reaction mixture to block remaining active sites. This blocking reaction was completed in 1 hour at 4°C with sample rotation. The matrix was washed with two 1.0 ml aliquots of 1.5 M NaCl. The supernatant from the coupling reaction and the two 1.5 M NaCl wash volumes combined and dialyzed against PBS at 4°C.

In order to determine the extent of coupling, the plasmin content of the dialyzed sample (determined by means of absorbance at 280 nm using an A_{1cm}^{280nm} value of 17.0) was compared with the known starting plasmin concentration. The efficiency of coupling was estimated to be 90%.

The Affi-Prep 10 - Plasmin was loaded into an HR 10/10 FPLC compatible column (Pharmacia). The affinity matrix was equilibrated in 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, 0.02% NaN_3 pH 7.2. When not in use, the column was stored at 4°C.

Affinity Purification of Plasmin Receptor

The Affi-Prep 10-Plasmin HR 10/10 column was attached to a Pharmacia FPLC chromatography system and equilibrated at room temperature in 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, 0.02% sodium azide pH 7.2. 1.0-2.0 ml of crude supernatant from the mutanolysin extraction of bacterial strain of 64/14 (prepared as described above) was loaded onto the column at a flow rate of 0.02 ml/min. The flow rate was increased to a 1.0 ml/min rate during the washing step using equilibration buffer (approx. 200 mls). The column was either eluted at 0.2 ml/min with a 50 ml linear gradient of 0.0 M - 0.1 M L-Lysine in equilibration buffer, or eluted in a single step using equilibration buffer containing 0.1 M L-Lysine. The absorbance at 280 nm was continuously monitored and 1.0 ml fractions were collected. After each affinity purification procedure the column was washed with 20 mls of 2.0 M NaCl, followed by 200 mls of equilibration buffer and stored at 4°C.

Plasminogen Activation Assay for Streptokinase

The following assay (Zolton and Mertz, 1972; Teger-Nilsson et al., 1977) to measure SK activity was carried out in microtiter plates. 20 μl aliquots of streptokinase standards (a dilution series for the purpose of

generating a standard curve), or the samples to be tested were placed into the microtiter wells in duplicate. To the 20 μl aliquots, 40 μl of 50 mM Tris, pH 7.5, was added. 30 μl of a freshly prepared solution of human Glu-plasminogen, 20 μg/ml in 0.01 mM Triton X-100, was then added to the well and allowed to incubate at 37°C for 15 min. 30 μl of substrate was then added. Substrate is prepared as follows: To 1 volume substrate (5 mg/ml S-2251 in water) add 3 volumes 1.77 M NaCl in 0.32 M Tris, pH 7.5, and one volume of water. The plate was then incubated at 37°C to allow substrate hydrolysis, and product production is measured at 405 nm. Control wells from which either SK and/or plasminogen were omitted were included. These controls will indicate whether there is any plasmin contamination in the plasminogen preparation or if the sample being tested has any proteolytic activity for the substrate that is not dependent on plasminogen activation.

Solid Phase Assay for Plasminogen Activators

Samples to be tested for plasminogen activator activity by this assay (Dr. K. Johnston, personal communication) were first resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membranes were then immersed in blocking buffer (10 mM Tris, pH 8.0 containing 0.5% Tween-20, 0.5 M NaCl and 1.0% bovine serum albumin) for at least one hour at room temperature. The substrate overlay is prepared as follows: To a 2.0% agarose solution (Bio Rad Richmond, CA) in 0.15 M phosphate buffered saline, pH 7.5 was equilibrated at 50°C, with the chromogenic substrate S-2251 at a concentration of 100 μg/ml. Human plasminogen free of plasmin activity, was then added to a final concentration of 20 μg/ml. The agarose-substrate-plasminogen solution was then applied to an ethanol washed glass slide slightly larger than the nitrocellulose membrane

template. The nitrocellulose membrane containing the sample was then drained of excess blocking buffer and overlayed on the agarose-plasminogen-substrate gel. It is important to ensure uniform contact between the nitrocellulose membrane and the gel. The nitrocellulose membrane was allowed to remain in contact with the agarose for at least one hour at 37°C; the time of incubation is dependent upon the concentration of plasminogen activator present. At the termination of incubation, the nitrocellulose membrane was removed from the agarose-plasminogen-substrate gel and immersed for 5 min in freshly prepared 0.1% sodium nitrite dissolved in 1.0 N HCl followed immediately by immersion for 5 min in 0.5% ammonium sulfamate dissolved in 1.0 N HCl. The membrane was then transferred to a solution containing 0.05% N-1-napthylethylenediamine in 47.5% ethanol and observed for the appearance of red bands indicative of plasmin activity. Plasminogen activators present on the nitrocellulose membrane will activate the plasminogen present in the agarose which will in turn cleave the S-2251 substrate incorporated in the agarose. The chromogenic cleavage product (paranitroaniline) appears yellow and deposits on the nitrocellulose membrane. The chemical treatment of the membranes as described above will convert the deposited yellow paranitroaniline to a red color and fix it to the membrane. Membranes can be stored under water at 4°C.

Preparation of Polyclonal Rabbit Anti-plasmin Receptor Protein Antibody

Mutanolysin extracted 41,000 dalton plasmin receptor protein was purified by gel electrophoreses on a 10% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. The single stained band was cut from the gel and equilibrated in PBS-azide. The location of the 41,000 dalton plasmin binding band was determined by the position of the stained band

in the gel, and by Western blotting a small strip of the gel to nitrocellulose, followed by blocking and probing with ^{125}I-plasmin, and autoradiography as described in the polyacrylamide gel electrophoreses and protein blotting section of the Methods. The stained 41,000 dalton band was cut from the gel, and a portion containing approximately 300 μg was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously at 6 sites on a rabbit. The rabbit was boosted eight times with the 41,000 dalton protein-polyacrylamide gel emulsified in Freund's incomplete adjuvant (approximately 200 μg per boost) during a 14 month period. Pre-immune and immune IgG fractions were prepared from rabbit sera by Protein A-Sepharose (Sigma) affinity chromatography.

Results

A variety of extraction procedures were compared and were to determine the optimal method for solubilizing functional plasmin receptor activity. The extractions were carried out on heat killed 64/14 as described in detail in the Methods section. The bacterial samples were washed thoroughly prior to treatment to minimize carry over of culture media and secreted products to the extraction samples. This would therefore reduce the likelihood of significant streptokinase contamination (with the possible exception of intracellular forms).

The extraction techniques included: (1) Lancefield acid and alkaline extractions; (2) a time course trypsin digestion under suboptimal conditions for enzyme activity (conditions previously shown to maximize homogeneity of type III Fc receptor extraction, Reis et al., 1985); (3) Triton X-100/osmotic shock/lysozyme treatment; (4)

acetone/Triton X-100 extractions; and (5) mutanolysin digestion. The supernatants from these solubilizations were screened for ^{125}I-plasmin binding by the dot-botting procedure in Methods. Aliquots of cell free supernatants prepared from the extractions were applied to nitrocellulose membrane in a dot-blot apparatus. Blocking and washing of the nitrocellulose membranes was carried out according to Methods. The nitrocellulose membranes were then probed with ^{125}I-plasmin, washed and autoradiographed.

The results of the screening of the various extracts and preparations are shown in Figure 4-1. Extraction with mutanolysin demonstrated the highest yield of soluble plasmin binding activity (see Figure 4-1, row B, column 3).

The size heterogeneity of the soluble plasmin receptor activity in the mutanolysin extract of strain 64/14 was assessed by electrophoresis of a 50 μl aliquot of the extract on both reducing and non-reducing SDS-polyacrylamide gels which were then stained with silver or electroblotted onto nitrocellulose and probed with ^{125}I-plasmin. The protein staining pattern of the mutanolysin extract is shown in lane 2 of Figure 4-2. Plasmin binding activity was concentrated predominately in a band with an M_r of approximately 41,000 daltons (see Figure 4-2, Panel B). One μg of purified group C streptokinase (M_r approx. 48,000 daltons) was electrophoresed as a positive control, (Figure 4.2, lane 1, Panel A; lane 2, Panel B). An aliquot of the control mutanolysin digestion mixture and containing all the reactants except the bacteria was analyzed by SDS-PAGE reveled no plasmin binding activity by Western blotting nor any significant stainable bands (data not shown).

The possible release of the secreted plasminogen activator (streptokinase), from the strain 64/14, during the extraction, would be a

Figure 4-1. Dot-blot analysis of solubilized plasmin binding activities. Samples to be tested for plasmin binding activity were immobilized on nitrocellulose in a Bio-dot blot apparatus. The nitrocellulose membrane was blocked as described in the Methods and probed with ^{125}I-plasmin. The blot was washed and autoradiographed at -70°C for 10 hours using Kodak X-AR-5 film and an intensifying screen. Row A: columns 2 through 5: Kabikinase 400, 300, 200, and 100 ng respectivly; column 6: trypsin extraction (60 min); column 7: trypsin enzyme control. Row B: column 1: lysozyme/detergent/shock extract; column 2: lysozyme/detergent/shock control; column 3: mutanolysin extract; column 4: mutanolysin enzyme control; column 5: hot-alkali extract; column 6: hot-acid extract; column 7: acetone/detergent extract.

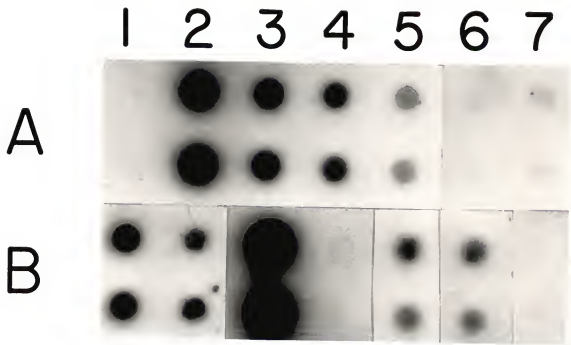
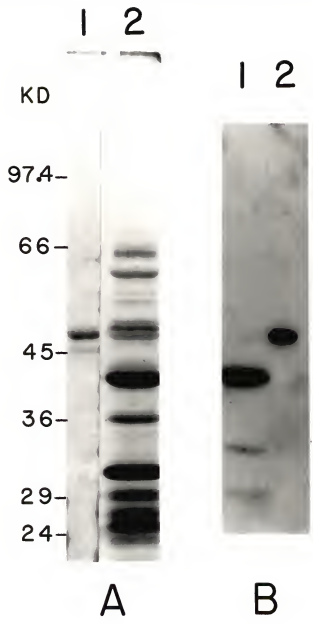

1 2 3 4 5 6 7
A
B

Figure 4-2. SDS-PAGE and Western blot analysis of mutanolysin extracted 64/14 bacterial plasmin binding activity. Parallel 10% SDS-polyacrylamide gels were electrophoresed. One gel was silver stained to detect protein molecules (Panel A) and the second was Western blotted and probed with ^{125}I-plasmin, as described in the Methods, and autoradiographed for 10 hours at -70°C with intensifying screens (Panel B). Panel A, lane 1: 1.0 μg of group C streptokinase (Kabikinase); lane 2: 50 μl of strain 64/14 mutanolysin extract. Panel B, lane 1: 50 μl of strain 64/14 mutanolysin extract; lane 2: 1.0 μg of group C streptokinase (Kabikinase).


1 2
KD
97.4
66
45
36
29
24
A
1 2
B

possible confusing factor in the isolation and characterization of the surface plasmin receptor. The production of streptokinase by streptococci is optimal when the pH of the growth medium is maintained at 7.0 to 8.0 (Johnston and Zabriskie, 1986). The maintenance of a pH above 6.8 prevents the activation of an extracellular zymogen to an active mercaptoproteinase (Elliott and Dole, 1947; Liu and Elliott, 1965) produced by the bacteria, which would significantly contribute to the proteolytic hydrolysis of secreted streptokinase. Therefore, solubilized plasmin receptor activity was prepared by mutanolysin extraction as before, but from bacteria harvested from chemically defined media cultures in which the pH was not allowed to become acidic. In addition, the supernatants from these cultures were collected, filtered and concentrated as a source of streptokinase from the strain 64/14 bacteria for comparative analyses.

The production of plasmin receptor from chemically defined media, pH controlled, cultures was first investigated. Receptor activity was expressed, mutanolysin extracted, and also affinity purified. Furthermore, there was no change in the molecular weight of the plasmin receptor.

The production of streptokinase from the strain 64/14 bacteria was measured functionally, by use of a quantitative plasminogen activation assay. The commercially available highly purified group C streptokinase (Kabikinase) (Kabivitrum, A.B., Stockholm, Sweden.) was used as a standard in these studies.

The fluid phase assay for plasminogen activator activity (see Methods for precise experimental details) was also used to measure plasminogen activator activity in the mutanolysin extraction preparations

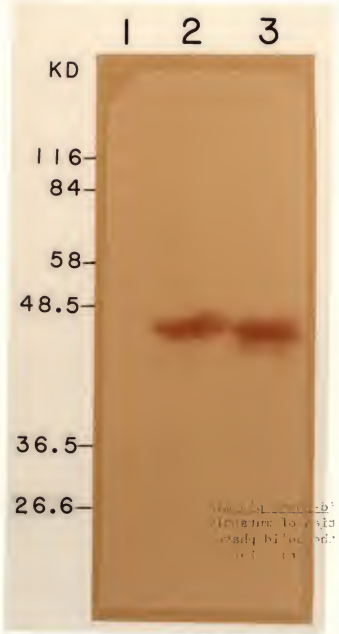
as well as the samples of strain 64/14 concentrated supernatant. The mutanolysin extracts of strain 64/14 were totally devoid of plasminogen activator activity (data not shown). The strain 64/14 concentrated supernatant was found to contain approximately 3,555 ± 72 units of SK activity per ml, or approximately 37 μg/ml of streptokinase, based on the kabikinase standard. There was a total of 15 mls of concentrated supernatant from 1 liter of this strain 64/14 bacterial culture. The possibility that treatment of streptokinase with mutanolysin may destroy plasminogen activator activity was considered, and under the conditions of mutanolysin treatment used for extraction no loss of plasminogen activator activity of mutanolysin treated streptokinase was observed (data not shown).

A second assay to investigate plasminogen activator activity was also used. This is a semi-quantitative, solid-phase assay designed to correlate plasminogen activator activity to molecular weight of the activator present in a sample (Dr. K. Johnston, personal communication). In this assay the samples to be analyzed are first electrophoresed by SDS-PAGE, separating individual proteins, and are then electroblotted onto nitrocellulose membrane (see Methods). The nitrocellulose membrane is then blocked with BSA, and applied to an agarose film containing plasminogen and the chromogenic substrate S-2251. The plasminogen activator present on the membrane will activate plasminogen in the agarose which will in turn hydrolyze the S-2251. After allowing a period of activation (time determined by the amount of plasminogen activator present in the sample) the paranitroaniline product that passively adheres to the membrane is chemically fixed to membrane. This fixation procedure results in the production of a pink band(s) indicative of the

presence of plasminogen activator. Samples of the mutanolysin extracted plasmin binding activity preparation, concentrated supernatant from the strain 64/14 pH controlled CDM cultures, and purified streptokinase (Kabikinase) as a positive control were all analyzed with this assay system. The results are shown in Figure 4-3. There were no bands of activator activity present in the mutanolysin extracts from the 64/14 bacteria. However, there was a streptokinase activity in the culture supernatant of this strain. This secreted plasminogen activator activity co-migrated at the same molecular weight (approx. 48,000 daltons) as the purified sample of group C streptokinase obtained from Kabivitrum.

Because of the high level of plasmin receptor activity in the extractions prepared by mutanolysin digestion, this preparation was chosen as material for further analysis and purification of the strain 64/14 plasmin receptor from bacteria grown in chemically defined media. Due to the simplicity and specificity offered by affinity chromatography, the plasmin receptor activity was purified from the mutanolysin extracts using a plasmin affinity matrix prepared as described in the Methods. Briefly, approximately 50 mg of purified human plasminogen was activated to plasmin by incubation in the presence of the plasminogen activator urokinase. The plasmin was then enzymatically inactivated and coupled to 6.0 mls of the affinity chromatography support Affi-Prep 10 (Bio Rad). Following ligand coupling, the remaining active sites on the matrix were blocked with 1.0 M ethanolamine HCl pH 8.0. The matrix was then washed with 2.0 M NaCl. The Affi-Prep 10 - inactivated plasmin was loaded intoan HR 10/10 FPLC compatible column (Pharmacia). The affinity matrix was again washed with 2.0 M NaCl, and then equilibrated in 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, and 0.02% NaN_3 pH 7.4.

Figure 4-3. Solid-phase plasminogen activation assay. The plasminogen activator activities of mutanolysin extracted and secreted proteins were monitored using the solid phase plasminogen activator assay (Johnston, personal communication). Lane 1: 50 μl of mutanolysin extracted 64/14 plasmin binding material (approx. 5-10 μg of 41,000 dalton band); Lane2: 60 μl of strain 64/14 concentrated supernatant (approx. 2.0 μg of streptokinase); Lane 3: 2.0 μg of group C streptokinase (Kabikinase). For precise experimental details see Methods.


1
2
3
KD
116
84
58
48.5
36.5
26.6

The Affi-Prep 10-plasmin HR 10/10 column was then either attached to a Pharmacia FPLC chromatography system and used at room temperature, or used at 4°C with a peristaltic pump and fraction collector (Pharmacia). The mutanolysin extract was dialyzed into 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, and 0.02% NaN_3 pH 7.4. prior to chromatography. 1.0-2.0 mls of cell free mutanolysin extract of strain 64/14 was routinely applied to the blocked plasmin affinity column matrix in 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, and 0.02% NaN_3 pH 7.4. After loading the extract onto the column, the matrix was washed with this buffer until the OD 280 nm returned to base line absorbance (approximately 20 mls). Bound plasmin receptor activity was eluted with either a 50 ml linear gradient of 0.0 - 0.1 M L-Lysine in 0.05 M Na_2HPO_4, 0.15 M NaCl, 1.0 mM benzamidine HCl, and 0.02% NaN_3 pH 7.4, or in a single step using buffer containing 0.1 M L-lysine. The absorbance at 280 nm was continuously monitored and 1.0 ml fractions were collected.

Fractions eluted from the affinity column were assayed by the dot blotting and ^{125}I-plasmin probing method. The plasmin binding functional activity eluted from the column correlated to absorbance at 280 nm (see Figure 4-4). This material was then analyzed by SDS-PAGE followed by either silver or Coomassie brilliant blue R-250 staining, and Western blotting and probing with iodinated plasmin. The results shown in Figure 4-5 indicate silver stained band at 41,000 daltons eluted from the affinity column with lysine. This band corresponds to the plasmin binding activity seen in the crude extract. The plasmin affinity purified material was then analyzed by Western blotting and probing with ^{125}I-plasmin. Figure 4-6, Panel A, shows another sample of plasmin affinity purified receptor, eluted using 0.1 M lysine in a single step.

Figure 4-4. Representative profile of an affinity purification of strain 64/14 mutanolysin extracted plasmin binding activity. One ml of a mutanolysin extract of strain 64/14 was applied to a column of immobilized enzymatically inactive plasmin. Bound material was eluted with a gradient of L-lysine. All fractions were screened for plasmin binding activity by dot-blot analysis. The resulting autoradiograph of dot-blotted fractions (50 μl) probed with ^{125}I-plasmin is shown for the coresponding fractions below the X-axis.

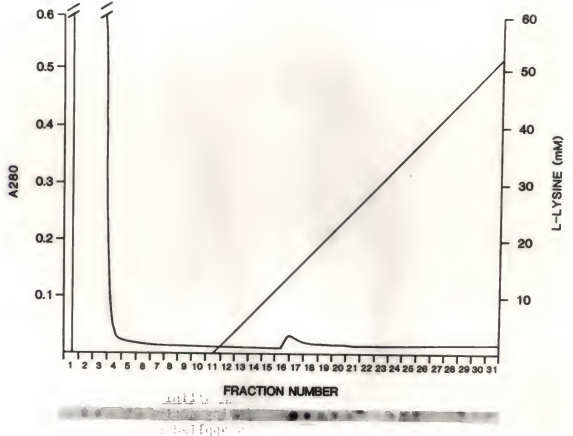
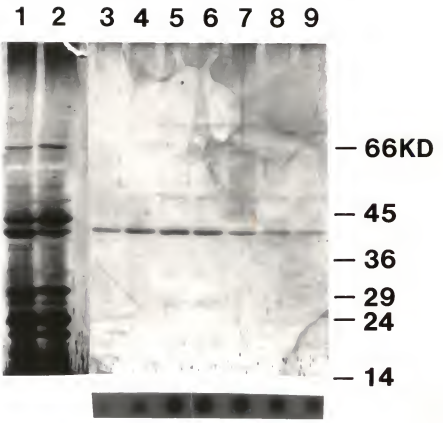

A280
0.6
0.5
0.4
0.3
0.2
0.1
L-LYSINE (mM)
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
FRACTION NUMBER

Figure 4-5. Analysis of affinity purified plasmin binding material from the strain 64/14 mutanolysin extract. 50 µl aliqouts of affinity purified fractions containing plasmin binding activity were electrophoresed on a 10% SDS-PAGE gel and stained with silver. The functional activity of each sample was monitored by dot-blot analysis and probing with ^{125}I-plasmin, and is shown below the coresponding lane on the SDS-polyacrylamide gel.


1 2 3 4 5 6 7 8 9
66KD
45
36
29
24
14

The activity eluted in three 1.0 ml fractions (Lanes 3-5 of Figure 4-6 Panel A). The greatest activity was found in fraction number two, corresponding to lane four of Figure 4-6, Panel A, and 50 μl of this fraction was analyzed by Western blotting. Figure 4-6, Panel B, is an autoradiograph of Western blotted extracted plasmin receptor preparation (Lane 1) and plasmin affinity purified receptor (Lane 3), which demonstrates that the 41,000 dalton molecule has retained functional plasmin binding activity following affinity purification. Treatment of the affinity purified material with trypsin destroys the ability of the 41,000 dalton molecule to bind plasmin and results in the disappearance of the 41,000 dalton stained band on SDS-polyacrylamide gel. These results clearly indicate the purification of a plasmin binding activity from a mutanolysin extract of the streptococcal strain 64/14 bacteria by means of affinity chromatography. Taken together these results indicate that the extracted surface receptor for human plasmin, and streptokinase produced by the strain 64/14 streptococcus, are physicochemically (molecular weight) and functionally (plasminogen activator activity) distinct molecules.

The isolated 41,000 dalton plasmin receptor protein was used to immunize a rabbit as described in the Methods. The resulting antibody was used to probe both the mutanolysin extract of strain 64/14, concentrated culture supernatant of strain 64/14, and group C streptokinase (Kabikinase) using a sandwich Western blot, and the results are shown in Figure 4-7.

Specific antigen-antibody complexes on the nitrocellulose were detected by probing with ^{125}I-Protein G. This antibody recognized only the 41,000 dalton band in the mutanolysin extract and a corresponding

Figure 4-6. SDS-PAGE and Western blot analysis of mutanolysin extracted, affinity purified plasmin binding activity. Parallel 10% SDS-polyacrylamide gels were electrophoresed. One gel was silver stained to detect the distribution of proteins present (Panel A). The proteins on the second gel were transfered to nitrocellulose by Western blotting and probed with ^{125}I-plasmin according to Methods. Panel B shows the results of the autoradiograph demonstrating functional activity. Panel A, lane M: molecular weight markers; lane 1: 50 μl of strain 64/14 mutanolysin extract; lane 2-4: three fractions containing lysine eluted plasmin binding activity. Panel B, lane 1: 50 μl of strain 64/14 mutanolysin extract; lane 3: 50 μl of the lysine eluted plasmin binding activity from the fraction shown in lane 3 of Panel A.

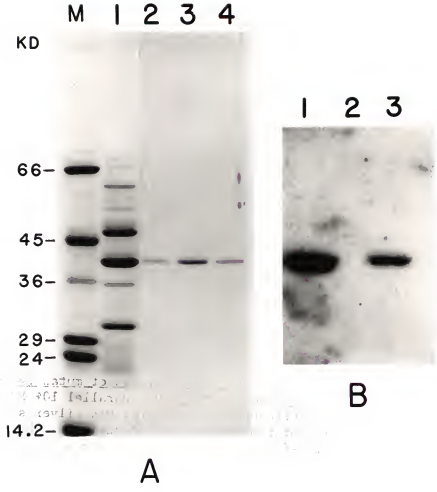
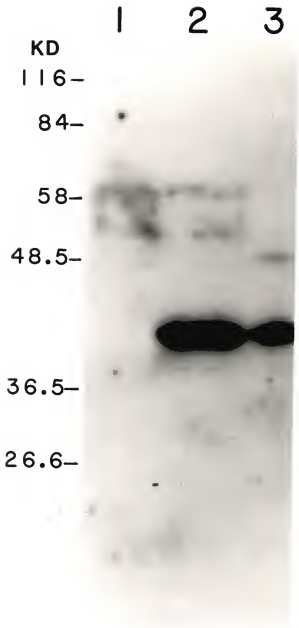

M 1 2 3 4
KD
66-
45-
36-
29-
24-
14.2-
A
1 2 3
B

Figure 4-7. SDS-PAGE and Western blot analysis of plasmin receptor protein with a polyclonal rabbit antibody. The isolated plasmin binding protein, streptokinase from the same group A strain, and group C streptokinase were compared for reactivity with a polyclonal rabbit antibody to the purified plasmin receptor molecule. Lane 1: 2.0 μg of group C streptokinase (Kabikinase); lane 2: 50 μl of extracted plasmin receptor preparation (approx. 5 μg of the 41,000 dalton molecule); lane 3: 60 μl of strain 64/14 concentrated supernatant (approx. 2.0 μg of 64/14 streptokinase). The proteins on the nitrocellulose blot were probed in a sandwich assay first with the polyclonal anti-plasmin receptor antibody followed by ^{125}I-Protein G as described in the Methods. The resulting blot was autoradiographed at -70°C for 10 hours with intensifying screens.


1
2
3
KD
116–
84–
58–
48.5–
36.5–
26.6–

protein in the concentrated culture supernatant of strain 64/14. Neither the 48,000 dalton plasminogen activator protein present in the concentrated culture supernatant of strain 64/14 (Figure 4-3), nor group C streptokinase was recognized by this antibody (Figure 4-7).

Discussion

Group A streptococci have been recognized for many years to secrete a protein, streptokinase, with a high affinity for both plasminogen and plasmin (Tillett and Garner, 1933). The experiments documented in Chapters Two and Three, have described a surface receptor on certain group A streptococci that displays selective binding activity towards plasmin, while having minimal reactivity with the zymogen form of the molecule, Glu-plasminogen. The purpose of the experiments described in this chapter were to isolate the plasmin receptor and compare it on a functional basis to the secreted streptokinase protein produced by the same group A streptococcal strain, 64/14.

A variety of different extraction techniques were compared and treatment with mutanolysin yielded the highest quantity of soluble plasmin binding activity. This activity was associated with a 41,000 dalton molecule by Western blot analysis, under both reducing and non-reducing conditions, and no evidence for subunit structure by intramolecular disulfide bonds was observed. This plasmin binding molecule was protein in nature, and was totally devoid of plasminogen activator activity. The 41,000 dalton plasmin receptor protein was purified from the mutanolysin extract of strain 64/14 by affinity chromatography using enzymatically inactivated immobilized human plasmin. Bound receptor activity on the column was specifically eluted with

L-lysine or EACA at the concentrations which reversibly inhibit plasmin binding to this streptococci (Chapter Two). The affinity purified 41,000 dalton protein was demonstrated to retain functional activity by Western blot analysis and probing with ^{125}I-plasmin.

The secreted protein, streptokinase, identified in the concentrated culture supernatant of strain 64/14 (M_r approximately 48,000) has the ability to bind plasminogen and once complexed to the zymogen, can act as a plasminogen activator converting plasminogen to plasmin. By contrast the cell bound plasmin receptor lacks plasminogen activator activity and demonstrates binding specificity towards plasmin rather than the native zymogen, Glu-plasminogen. The secretion of streptokinase into an environment containing plasminogen would result in plasminogen activation and the generation of plasmin. Plasmin generated by this reaction could then bind specifically to the receptor on the surface of the bacteria. In experiments documented in Chapter Two, it was recognized that once plasmin bound to the bacterial surface receptor it retained enzymatic activity. Furthermore, this cell bound plasmin activity could not be regulated by plasmin's normal physiological inhibitor α_2-antiplasmin.

The ability of bacteria, not only to produce plasminogen activators, but to associate the enzymatically active product on their cell surface in an physiologically nonregulatable form may prove to be an important factor in the ability of these pathogens to invade human tissue. The expression of two functionally distinct streptococcal proteins with affinity for proteins of the human fibrinolytic system is intriguing. In the next chapter, the relationship between the plasmin receptor and streptokinase protein produced by strain 64/14 is compared physicochemically, functionally, and antigenically.

CHAPTER FIVE
COMPARISON OF THE GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN PLASMIN WITH STREPTOKINASE

Introduction

In the previous chapter I have described the isolation of a specific receptor for human plasmin from a group A streptococcus. This was achieved by first solubilizing the receptor by treatment with the enzyme mutanolysin followed by affinity purification on a column of immobilized plasmin. The purified functionally active receptor material had a M_r of approximately 41,000 daltons as measured by SDS-polyacrylamide gel electrophoresis and lacked plasminogen activator activity. These findings indicate that the plasmin receptor protein was not an intact streptokinase molecule. The purpose of the studies presented in this chapter were to perform a more complete comparison of the group A streptococcal receptor for human plasmin and streptokinase with respect to plasminogen activator activity; their binding specificities for domains of the plasmin molecule; and examine possible antigenic relatedness.

The results presented in this chapter demonstrate that the plasmin receptor and streptokinase, while both produced by the same strain of group A streptococci bacteria and having high affinity for plasmin, are physicochemically, functionally, and antigenically distinct molecules.

Materials and Methods

Materials

Nitrocellulose was purchased from BioRad, Richmond CA. Affinity purified goat anti-mouse IgG, heavy and light chains specific, was purchased from Cappel, Organon Teknika Corp. West Chester, PA. Ribi Adjuvant System (RAS) was purchased from Ribi Immunochem Research Inc. Hamilton, MT. Mouse anti-group C streptokinase monoclonal antibodies were a gift from Dr. K. Johnston. All other chemicals and reagents were purchased from Sigma Chemical Co. St. Louis, MO.

Bacterial Strains

The Lancefield group A β hemolytic streptococcal strains 64/14, B923, and A995; and the Lancefield group C β hemolytic streptococcal strain 26RP66 and the ATCC strain 12449 were grown as stationary cultures at 37°C for 24 to 36 hrs, in one to two liter batches of a chemically defined media described by Van De Rijn and Kessler (1980), containing 0.1% phenol red. The pH of the culture was maintained above 7.0, as monitored by the indicator dye. Approximately 3.0 to 5.0 g (wet weight) of bacteria could be recovered per liter of media. Bacteria were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.02% sodium azide. The bacteria were heat killed at 80°C for 15 min. The suspension was centrifuged and the pellet washed three times with PBS containing 0.02% sodium azide. Aliquots could be stored at -20°C, or used immediately for extraction purposes.

Bacterial Culture Supernatants

Culture supernatants from the various strains of bacteria were recovered following removal of the bacteria by centrifugation. The supernatants were filtered through a 0.22 μm filter and immediately

concentrated using an Amicon concentrator (Amicon Corp., Danvers, MA) fitted with a YM-10 membrane at 4°C. Supernatants were concentrated approximately 100 fold, and aliquoted in 0.5 ml fractions flash frozen and stored at -70°C.

Radioiodination of Proteins

Human plasminogen, isolated plasmin heavy chain, and isolated plasmin light chain were iodinated by the chloramine T method using Iodobeads (Pierce Chem. Co., Rockford, IL) as described by Markwell (1982). The labeled proteins were separated from free iodine by passage over a G25 column (PD-10 Pharmacia) and collected in 0.15 M Veronal buffered saline pH 7.35 containing 0.001 M Mg^{++}, 0.00015 M Ca^{++} and 0.1% gelatin (VBS-gel). The labeled proteins were stored in aliquots containing 0.02% sodium azide at -20°C. Labeled aliquots were used once and discarded.

Generation of Plasmin

Plasmin was generated from either radiolabeled or unlabeled plasminogen by reaction with urokinase. Three μl of urokinase (Sigma 20 u/ml) was added to a 400 μl solution of 1 μM plasminogen containing 0.04 M lysine. The mixture was incubated at 37°C for 45 minutes unless stated otherwise. The efficiency of plasmin generation was followed by measuring the conversion of the single chain plasminogen molecule (M_r=90,000 daltons) into heavy chains (M_r=60,000 daltons) and light chains (M_r=25,000 daltons) as determined by the migration of radiolabeled proteins, following denaturation and reduction, on 10% SDS-polyacrylamide gels. The migration of labeled proteins was determined by autoradiographic exposure of dried gels to Kodak XAR-5 film with intensifying screens at -70°C for 15-20 hours. The integrity of the iodinated plasmin

heavy and light chains preparations were examined by SDS-PAGE and autoradiography in a similar manner.

Polyacrylamide Gel Electrophoresis and Protein Blotting

Electrophoresis was carried out as described by Laemmli (1970). Polyacrylamide separating gels were 10% and contained 0.1% sodium dodecylsulfate (SDS), 0.375 M Tris at pH 8.8. Stacking gels were 4% and contained 0.1% SDS and 0.125 M Tris at pH 6.8. Electrode buffer was 0.024 M Tris, 0.192 M glycine, 0.1% SDS at pH 8.3 Samples were diluted 1:2 with sample buffer containing 0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue and heated at 80-90°C for 3 minutes. Gels were run at 45 volts constant voltage for approximately 15-18 hours. Slab gels were used in the Bio-Rad Protean II system (BioRad, Richmond, CA). Molecular weight markers were run on all gels. Gels intended for Western blot transfer contained pre-stained markers (Sigma) applied as a mixture which included: triosephosphate isomerase (26,600), lactic dehydrogenase (36,500), fumarase (48,500), pyruvate kinase (58,000), fructase-6-phosphate kinase (84,000), β-galactosidase (116,000), and alpha 2-macroglobulin (180,000). After electrophoresis, gels intended for Western blotting were equilibrated in 25 mM Tris, 0.2 M glycine pH 8.0 containing 20% v/v methanol (electroblot buffer) for 25 minutes. Protein blotting, from SDS-PAGE gels, was performed using the 'Trans-Blot SD Semi-Dry' electrophoretic transfer cell (Bio Rad, Richmond, CA). Nitrocellulose transfer medium, also equilibrated in electroblot buffer, was sandwiched between the gel and two sheets of Whatman 3 mm paper. The gel was also backed with two sheets of 3 mm paper. Blots were blocked by washing a total of four times in 200-250 ml of 5.0 mM sodium diethylbarbiturate, 0.14 M NaCl,

0.5% gelatin, 0.15% Tween 20, 0.004% NaN_3 pH 7.35 (blotting wash buffer I) for 15 minutes per wash. Blots were then probed for 3-4 hours at room temperature while rotating in one of the following probing solutions: For probing with either plasmin, plasmin-EACA, plasmin heavy chain, or plasmin light chain, blots were probed with blotting wash buffer I containing 2.0 mM PMSF and ^{125}I-labeled plasmin at 2 x 10^5-3 x 10^5 cpm/ml with or without 1.0 mM EACA, or blotting wash buffer I containing 2.0 mM PMSF and ^{125}I-plasmin heavy or light chain at 2 x 10^5-3 x 10^5 cpm/ml. For probing with rabbit, anti-plasmin receptor antibody or anti-group C streptokinase antibody, blots were probed with blotting wash buffer I containing 4.3 μg IgG per ml of probing solution (approximately a 1:3000 dilution of antisera) for three hours, washed twice for 20 min with 300 mls of blotting wash buffer I, and probed with ^{125}I-Protein G at 2 x 10^5-3 x 10^5 cpm/ml. For probing with mouse, anti-group C streptokinase monoclonal antibodies, blots were probed with blotting wash buffer I containing a 1:100 dilution of the stock solutions for three hours, washed twice for 20 min with 300 mls of blotting wash buffer I, followed by probing for three hours with blotting wash buffer I containing goat, anti-mouse IgG, antibody (Cappel) at 1.0 μg/ml, washed twice for 20 min with 300 mls of blotting wash buffer I, followed by probing with blotting wash buffer I containing ^{125}I-Protein G at 2 x 10^5-3 x 10^5 cpm/ml. Following the last probing step all blots were washed four times in 200-300 mls of 0.01 M EDTA, 1.0 M NaCl 0.25% gelatin, 0.15% Tween 20 for 20 minutes per wash. All washing and probing steps were carried out at ambient temperature. The probed, washed blots were air dried.

Autoradiographs were prepared by exposing the nitrocellulose blots to Kodak XAR-5 film with an intensifying screen for 15-24 hours at -70°C followed by automated film developing.

Molecular weight determinations on Western blots were made possible by the transfer of the prestained molecular markers.

For staining, gels were fixed in a solution of 40% ethanol and 10% acetic acid, stained with Coomassie brilliant blue R-250 (0.25% w/v in 40% ethanol and 10% acetic acid) for 1 hour, and destained by soaking in several changes of 10% ethanol and 10% acetic acid containing a small quantity of DE 52 (Whatman, England) as a dye adsorbent. All other gels prepared for staining were silver stained according to the procedure of Merril et al., (1981).

Solid Phase Assay for Plasminogen Activator Activity

Samples to be tested for plasminogen activator activity by this assay (Dr. K. Johnston, personal communication) are first resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membranes are then immersed in blocking buffer (10 mM Tris, pH 8.0 containing 0.5% Tween-20, 0.5 M NaCl and 1.0% bovine serum albumin) for at least one hour at room temperature. The substrate overlay is prepared as follows: To a 2.0% agarose solution (Bio Rad Richmond, CA) in 0.15 M phosphate buffered saline, pH 7.5 was equilibrated at 50°C, with the chromogenic substrate S-2251 at a concentration of 100 μg/ml. Human plasminogen free of plasmin activity, is then added to a final concentration of 20 μg/ml. The agarose-substrate-plasminogen solution is then applied to an ethanol washed glass slide slightly larger than the nitrocellulose membrane template. The nitrocellulose membrane containing the sample is then drained of excess blocking buffer and overlayed on the agarose-plasminogen-substrate gel. It is important to ensure uniform contact between the nitrocellulose membrane and the gel. The nitrocellulose membrane is allowed to remain in contact with the agarose for at least

one hour at 37°C; the time of incubation is dependent upon the concentration of plasminogen activator present. At the termination of incubation, the nitrocellulose membrane is removed from the agarose-plasminogen-substrate gel and immersed for 5 min in freshly prepared 0.1% sodium nitrite dissolved in 1.0 N HCl followed immediately by immersion for 5 min in 0.5% ammonium sulfamate dissolved in 1.0 N HCl. The membrane is then transferred to a solution containing 0.05% N-1-napthylethylenediamine in 47.5% ethanol and observed for the appearance of red bands indicative of plasmin activity. Plasminogen activators present on the nitrocellulose membrane will activate the plasminogen present in the agarose which will in turn cleave the S-2251 substrate incorporated in the agarose. The chromogenic cleavage product (paranitroaniline) appears yellow and deposits on the nitrocellulose membrane. The chemical treatment of the membranes as described above will convert the deposited yellow paranitroaniline to a red color and fix it to the membrane. Membranes can be stored under water at 4°C.

Production of Rabbit Anti-streptokinase and Rabbit Anti-plasmin Receptor Polyclonal Antibodies

Highly purified streptokinase from a Group C streptococcus was obtained from KabiVitrum A.B., Stockholm, Sweden. Approximately 1.38 mg of the antigen was electrophoresed on a 7.5% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. The single stained band was cut from the gel and equilibrated in PBS-Azide. A portion of the gel slice containing approximately 345 μg of streptokinase was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously at 6 sites on a rabbit. The rabbit was boosted three times at two week intervals with the streptokinase polyacrylamide gel emulsified in Freund's incomplete adjuvant (approximately 300 μg per

boost). Generation of polyclonal rabbit anti-plasmin receptor 41,000 dalton protein was prepared in a similar manner using the strain 64/14 mutanolysin extracted plasmin receptor preparation, except that the 41,000 dalton protein was separated on 10% SDS-PAGE gels and used in 150 μg to 200 μg aliquots for immunization. Pre-immune and immune IgG fractions were prepared from rabbit sera by Protein A-Sepharose (Sigma) affinity chromatography.

Production of Mouse Anti-plasmin Receptor Polyclonal Antibody

Plasmin receptor protein (41,000 dalton molecule) was gel purified on 10% SDS-polyacrylamide gels under reducing conditions as described above. Following electrophoresis each slab gel was then Western-blotted to a sheet of nitrocellulose membrane. A small vertical strip from each nitrocellulose blot was cut and probed with ^{125}I-plasmin as described above and autoradiographed to confirm the position of the 41,000 dalton plasmin binding protein. The remainder of each nitrocellulose sheet was then stained with 1.0% Fast Green according to the procedure described by Chiles et al., (1987) and the position of the 41,000 dalton band located and aligned with the autoradiographed strip. The marked band on each nitrocellulose sheet was then carefully cut out to avoid any contamination, and divided into four equal fractions containing approximately 500 μg of protein. The strips were then equilibrated in PBS and sonicated to a fine powder. The immunization schedule was as follows: Four groups of 6-8 week old out-bred female mice were used with ten mice per group. Part I (initial inoculation) groups 1+3: Each mouse in these groups was injected in two sites with (100μl/25μg) of sonicated nitrocellulose-Ag in complete Freund's Adjuvant subcutaneously. Group 2: This group was injected in two sites with (100μl/25μg) of sonicated

nitrocellulose-Ag in a non-Freund's adjuvant (T1501). The vehicle of this adjuvant has been described by Woodard and Jasman (1985), and the adjuvant (T1501) has been described by Hunter and Bennett (1984). The use of this combination was developed by Woodard and Jasman. Group 4: This group was injected with (100μl/50μg) of sonicated nitrocellulose-Ag in Ribi Adjuvant intraperitoneally. Part II first boost (2 weeks after initial inoculation): Mice in Groups 1+3 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag in incomplete Freund's Adjuvant in two sites subcutaneously. Mice in group 2 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag in the non-Freund's adjuvant (Woodard and Jasman, 1985) subcutaneously. Mice in group 4 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag in Ribi Adjuvant intraperitoneally. Part III second boost (4 weeks after initial inoculation): Mice in group 1 were boosted with (100μl/10μg) of solubilized nitrocellulose-Ag in dimethylsulfoxide intraperitoneally. Mice in group 2 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag in ddH_2O plus glycogen intraperitoneally. Mice in group 3 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag plus glycogen intraperitoneally. Mice in group 4 were boosted with (100μl/10μg) of sonicated nitrocellulose-Ag in ddH_2O intraperitoneally. Four days after the final boost all mice were sacrificed and ascites harvested. The peritoneal cavities were washed with PBS and also harvested, and the resulting fluids from each group pooled and stored as aliquots at -70°C, with 0.02% sodium azide, until used.

Results

Streptokinase from three group A Streptococcal strains (64/14, B923 and A995), and two group C streptococcal strains (ATCC 12449 and 26RP66),

and the commercially available purified Kabikinase (KabiVitrum, Sockholm, Sweden) were compared physicochemically, functionally and antigenically. The well characterized highly purified commercially available group C streptokinase (Kabikinase) was used as a reference. Streptokinase proteins were obtained from the bacterial strains by growing them in chemically defined media under pH controlled conditions in order to optimize the yield of streptokinase in the culture supernatants (Johnston and Zabriskie, 1986). The quantity of functionally active streptokinase was measured using the fluid phase plasminogen activator assay described in the Methods. This assay used the highly purified streptokinase (Kabikinase) as a standard. The quantities of streptokinase produced by the various strains of either group A or C streptococcal bacteria are summarized in Table 5-1. The concentrations of each of the plasminogen activators (SK)s were expressed as units relative to Kabikinase.

We have previously demonstrated that the binding of human plasmin to the group A streptococcal strain 64/14 was both inhibitable and reversible with L-Lysine or EACA. Furthermore, the binding of human plasmin to the extracted receptor from this bacteria was also shown to be inhibitable and reversible with these molecules in Western and dot-blot assays using ^{125}I-plasmin as the probe. However, it was demonstrated that the binding of human plasmin to group C streptokinase (Kabikinase) was not sensitive to L-Lysine or EACA in similar assays. The following series of experiments were designed to compare the binding specificities of the extracted plasmin receptor from strain 64/14 to the streptokinase produced by strain 64/14 from the same culture, as well as those from two other group A streptococci, two group C streptococci and Kabikinase. Shown in Figure 5-1 are parallel 10% SDS-PAGE Western blots of extracted

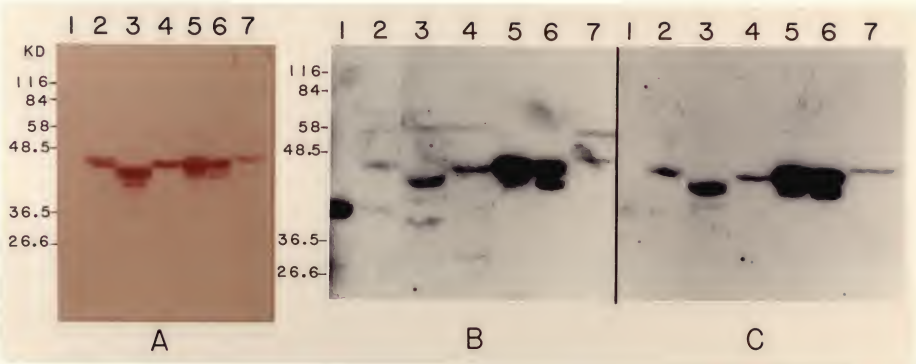
Table 5-1.

Fluid-phase plasminogen activator activity assay.

	Strain	Plasminogen Activator Activity* Units/ml	approx. μg/ml
Group A Strep.	64/14	3,550±70	40
	B923	3,970±60	40
	A995	3,250±60	30
Group C Strep.	26RP66	2,330±80	20
	ATCC 12449	44,000±170	460
64/14 mutanolysin extract		50±4	0.5

*Plasminogen activator activity (e.g. streptokinase) present in concentrated culture supernatants, and mutanolysin extracted plasmin binding activity. Estimates were determined using purified Kabikinase as the standard. See Methods for precise experimental details.

Figure 5-1. Functional identification and distinction of streptokinase proteins and plasmin binding receptor protein. Parallel 10% SDS-polyacrylamide gels were electrophoresed. The proteins on one gel were transferred to nitrocellulose, and the membrane blocked and used in the solid-phase plasminogen activation assay (Panel A) as described in the Methods. The proteins in the second and third gel were transferred to separate nitrocellulose sheets, and the membranes blocked and probed with ^{125}I-plasmin (Panel B) and ^{125}I-plasmin in the presence of 1.0 mM EACA (Panel C) as described in the Methods. The resulting blots (Panels B and C) were autoradiographed at -70°C for 24 hours with intensifying screens. Lane(s) 1: exracted plasmin receptor preparation (approx. 5.0 μg); lane(s) 2: approx. 2.0 μg of 64/14 SK; lane(s) 3: approx. 2.0 μg of B923 SK; lane(s) 4: approx. 1.0 μg of A995 SK; lane(s) 5: approx. 2.0 μg of Kabikinase; lane(s) 6: approx. 2.0 μg of ATCC 12449 SK; lane(s) 7: approx. 1.0 μg of 26RP66 SK. Streptokinase proteins from the strains 64/14, B923, A995, ATCC 12449, and 26RP66 were contained in concentrated culture supernatants, see Methods for precise experimental details.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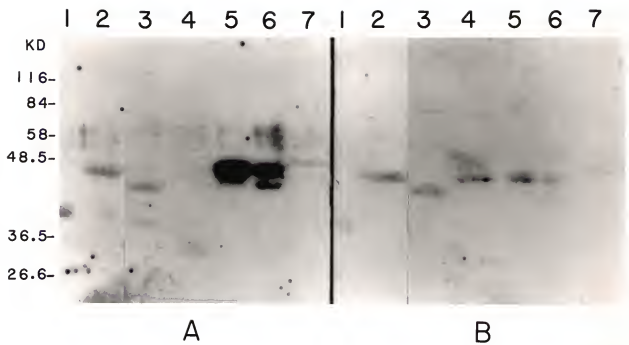
plasmin receptor and concentrated culture supernatants, which contain streptokinase, from the strains of streptococcal bacteria tested. Panel A in Figure 5-1 identifies the molecular species of streptokinase by means of plasminogen activator potential using a solid phase assay as described in the Methods. The major species of streptokinase produced by all but group A B923 had a molecular weight of approximately 48,000 daltons. The major streptokinase molecular species from strain B923 was slightly smaller, approximately 46,000 daltons. There was no plasminogen activator activity in the extracted plasmin receptor preparation. Smaller molecular species of a given streptokinase, as seen in strain B923, ATCC 12449, and purified Kabikinase, result from degradation of the larger secreted protein (Johnston and Zabriskie, 1986). The Western blot depicted in Panel B of Figure 5-1 show the ^{125}I-plasmin binding activities and demonstrate the major binding activity in the various culture supernatants is associated with streptokinase, compare Panel A to B. Panel C of Figure 5-1 shows the ^{125}I-plasmin binding results in the presence of EACA, which is known to disrupt the binding of human plasmin to the 41,000 dalton plasmin receptor. These results indicate that all of the streptokinases appear to have a binding activity with human plasmin that is not disrupted in the presence of EACA. However, the 41,000 dalton extracted plasmin receptor is clearly shown to be sensitive to the presence of EACA. Compare lane 1 in Panels B and C of Figure 5-1.

The studies presented in Chapter Three demonstrated that unlike the group C streptokinase (Kabikinase), which has been shown to associate with plasmin through interactions with determinants located in the light chain of the plasmin molecule, the plasmin receptor associated with the

surface of the strain 64/14 streptococci possesses a plasmin binding activity which appears to be associated with determinants present in the intact heavy chain of plasmin in a conformationally dependent manner. I therefore examined the binding specificities for ^{125}I-labeled plasmin heavy chain and ^{125}I-labeled plasmin Light chain, by Western blot analysis, of the extracted plasmin receptor and streptokinase from strain 64/14, as well as the other streptokinases. The results presented in Figure 5-2 depict the binding reactivities of extracted plasmin receptor and the streptokinases from the strains of streptococci described above ^{125}I-plasmin heavy chain Panel A, and ^{125}I-plasmin light chain Panel B. The data clearly indicates that the group C streptokinase (Kabikinase), in agreement with earlier findings (Summaria and Robbins, 1976) has a much stronger reactivity with plasmin light chain than with plasmin heavy chain. The streptokinase from the group C strain ATCC 12449 also shows preferential reactivity for plasmin light chain. However, streptokinase proteins associated with 26RP66 and all three of the group A strains demonstrated equivalent reactivity with plasmin heavy chain and light chain. The reactivity of the plasmin receptor for either heavy chain or light chain was relatively weak. This binding is consistent with my earlier observations (Chapter Three) which indicated that for optimal binding reactivity of the bacterial plasmin receptor there appeared to be a requirement for a specific conformation that was best represented in the intact plasmin molecule or the intact, conformationally modified, zymogen, Lys-plasminogen.

These results and the previous findings (Chapters Two, Three, and Four) provide evidence that the plasmin receptor and streptokinase are physicochemically and functionally distinct molecules. The

Figure 5-2. Comparison of binding reactivities of streptokinase proteins and plasmin binding receptor protein with ^{125}I-plasmin heavy chain and ^{125}I-plasmin light chain. Parallel 10% SDS-polyacrylamide gels were electrophoresed. The proteins on each gel were transferred to separate nitrocellulose sheets and the membranes blocked and probed with ^{125}I-plasmin heavy chain (Panel A) or ^{125}I-plasmin light chain (Panel B) as described in the Methods. The resulting blots were autoradiographed at -70°C for 24 hours with intensifying screens. Lane(s) 1: exracted plasmin receptor preparation (approx. 5.0 μg); lane(s) 2: approx. 2.0 μg of 64/14 SK; lane(s) 3: approx. 2.0 μg of B923 SK; lane(s) 4: approx. 1.0 μg of A995 SK; lane(s) 5: approx. 2.0 μg of Kabikinase; lane(s) 6: approx. 2.0 μg of ATCC 12449 SK; lane(s) 7: approx. 1.0 μg of 26RP66 SK. Streptokinase proteins from the strains 64/14, B923, A995, ATCC 12449, and 26RP66 were contained in concentrated culture supernatants, see Methods for precise experimental details.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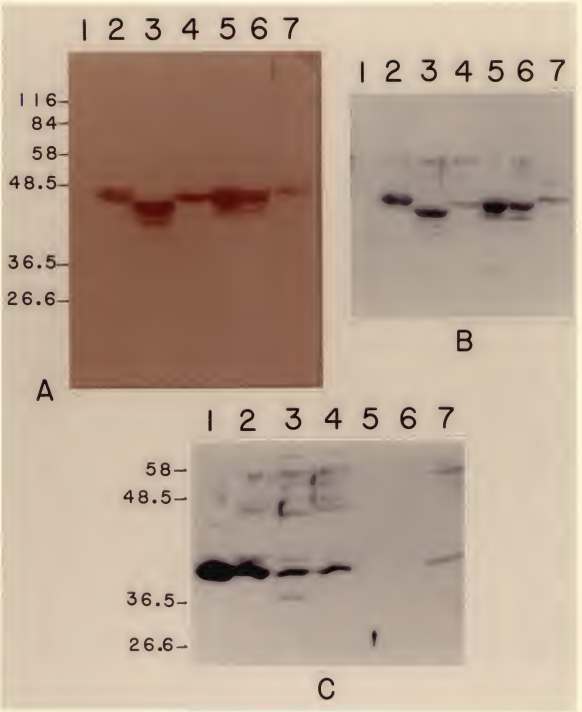
possibility that the two proteins arose from a common precursor and contained at least some common antigenic determinants was considered. This possibility was examined using a series of polyclonal and monoclonal antibodies to streptokinase, or rabbit and mouse polyclonal antibodies to the isolated strain 64/14 plasmin receptor.

A polyclonal rabbit antibody to group C streptokinase (Kabikinase) was prepared. This antibody could successfully be used in an ELISA assay system to measure streptokinase in culture supernatants of both group C and group A streptococcal bacteria (Reis et al., 1988). This polyclonal rabbit antibody to the extracted plasmin receptor was prepared using the same immunization procedure, see Methods for precise details. Using these polyclonal antibodies the antigenic relationship of the strain 64/14 plasmin receptor to the streptokinase produced by strain 64/14 as well as the other streptokinases examined was assessed by SDS-PAGE and Western blotting; probing with the two different polyclonal antibodies followed by ^{125}I-labeled Protein G. The results of these experiments are presented in Figure 5-3. Panel A of Figure 5-3 shows that the polyclonal rabbit anti-group C streptokinase antibody could detect efficiently all the streptokinases examined in concentrated culture supernatants from all the streptococcal isolates tested including the group A strain 64/14. The amount of streptokinase in each lane was approximately 1-2 μg, as measured by the fluid phase plasminogen activator assay. No reactivity was seen in the lane containing the 41,000 dalton extracted plasmin receptor protein. The receptor lanes contained approximately 5 μg of the 41,000 dalton protein, and was readily detected when a parallel gel was probed with the polyclonal rabbit anti-plasmin receptor antibody (see Panel B of Figure 5-3). This

Figure 5-3. Analysis of the antigenic relationship of the 64/14 plasmin receptor and streptokinase proteins. Parallel 10% SDS-polyacrylamide gels were electrophoresed. The proteins in each gel were transferred to separate nitrocellulose sheets, and one membrane was used in the solid-phase plasminogen activator assay (Panel A), to identify the molecular species of plasminogen activator molecules, as described in the Methods. The second and third membranes were probed in a sandwich assay first with polyclonal rabbit anti-group C streptokinase IgG (Panel B) or polyclonal rabbit anti-plasmin receptor IgG (Panel C), followed by ^{125}I-Protein G, as described in the Methods. The resulting blots (Panels B and C) were autoradiographed for 6 hours at -70°C with intensifying screens. Lane(s) 1: exracted plasmin receptor preparation (approx. 5.0 μg); lane(s) 2: approx. 2.0 μg of 64/14 SK; lane(s) 3: approx. 2.0 μg of B923 SK; lane(s) 4: approx. 1.0 μg of A995 SK; lane(s) 5: approx. 2.0 μg of Kabikinase; lane(s) 6: approx. 2.0 μg of ATCC 12449 SK; lane(s) 7: approx. 1.0 μg of 26RP66 SK. Streptokinase proteins from the strains 64/14, B923, A995, ATCC 12449, and 26RP66 were contained in concentrated culture supernatants, see Methods for precise experimental details.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polyclonal anti-plasmin receptor antibody could detect the 41,000 dalton protein in the extracted plasmin receptor preparation, as well as in the culture supernatants of all three group A streptococcal strains tested. However, this antibody did not detect the 48,000 dalton streptokinase protein in any of the culture supernatants including strain 64/14.

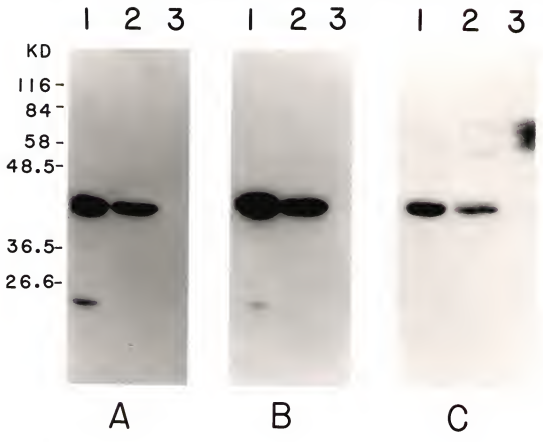
On prolonged exposure of the Western blots probed with the anti-receptor antibody, minor bands of reactivity could be seen at in the range of M_r 48,000 to 55,000 daltons in the culture supernatants from these bacteria. There was concern that the rabbit immunized to prepare the anti-plasmin receptor antibody may have previously had a streptococcal infection and therefore pre-existing antibody to streptococcal proteins. These reactive bands might be attributable to Fc-binding proteins produced by the group A (Type II a+b) (Yarnall and Boyle, 1986a) and group C (Type III) Fc-receptor proteins (Boyle and Reis, 1987). In similar experiments using normal rabbit IgG a similar pattern of reactive bands were observed when the autoradiographs were over-exposed, suggesting that these reactivities were not related to antigen specific interactions with rabbit immune antibody (data not shown).

In an attempt to eliminate these background reactivities, I prepared a polyclonal mouse anti-plasmin receptor antibody since the frequency of mouse anti-streptococcal antibodies in non-immune populations is less than observed in non-immune rabbits. Furthermore, mouse IgG reacts very poorly with Type II Fc-receptor (Yarnall and Boyle, 1986b). The polyclonal mouse anti-plasmin receptor antibodies were prepared as described in the Methods. Four immunization protocols were performed on separate groups of outbred mice, all involved the use of the

41,000 dalton protein-nitrocellulose in a powder form with an adjuvant (see Methods for precise details). Following the immunization schedule, ascites fluid and a PBS wash of the pleural cavities were obtained from each mouse and the material pooled by group.

The amount of mouse IgG in the ascites and washes of the four groups were first assayed by serial dilution and dot-blotting, followed by probing with goat anti-mouse IgG, followed by ^{125}I-Protein G. This was performed to equalize the amount of ascites or wash among the four groups of mice to be used later. The ascites and pleural washes of the four groups of mice were all tested against Western blotted extracted plasmin receptor from strain 64/14, 64/14 concentrated supernatant containing streptokinase, and the highly purified commercial group C streptokinase (Kabikinase). Blots were probed in a sandwich assay with mouse antibody, followed by goat anti-mouse IgG, followed by ^{125}I-Protein G. All four groups of mice showed reactivity with the 41,000 dalton protein. Two of the four groups had a much higher titer based on the signal intensity seen on the autoradiograph of the Western blot. Results using these two, higher titer, polyclonal mouse anti-plasmin receptor antibodies on Western blots are depicted in Figure 5-4. These data confirm the previous observations with the polyclonal rabbit anti-plasmin receptor antibody. The mouse IgG in the ascites fluid clearly shows strong reactivity with the 41,000 dalton protein in the extracted plasmin receptor preparation, lane 1 of panels A and B, as well as in the 64/14 concentrated supernatant, lane 2 of panels A and B. However, no significant reactivity is seen at 48,000 dalton position in the 64/14 concentrated supernatant or in the lane containing group C streptokinase (Kabikinase), lanes 2 and 3 of panels A and B, respectively. Panel C of

Figure 5-4. Analysis of the antigenic relationship of the 64/14 plasmin receptor and 64/14 streptokinase and group C streptokinase with mouse polyclonal anti-plasmin receptor antibodies. Parallel protein samples were electrophoresed in a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes, and the membranes probed in sandwich assays first with group II polyclonal mouse, anti-plasmin receptor acities fluid (Panel A) or with group IV polyclonal mouse, anti-plasmin receptor acities fluid (Panel B) or with polyclonal rabbit, anti-plasmin receptor IgG, followed by goat, anti-mouse IgG antibody (Panels A and B), followed by ^{125}I-Protein G. The resulting blots were auto-radiographed at -70°C for 6 hours with intensifying screens. Lane(s) 1: approx. 5.0 μg of plasmin receptor protein; lane(s) 2: concentrated 64/14 culture supernatant containing approx. 2.0 μg of 64/14 streptokinase; lane(s) 3: 4.0 μg of group C streptokinase. See Methods for precise experimental details.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84-
58-
48.5-
36.5-
26.6-
A
B
C

Figure 5-4 shows the reactivity of the rabbit polyclonal anti-plasmin receptor protein for comparison. These data clearly demonstrate that streptokinase and the bacterial plasmin receptor do not contain any common immunodominant epitopes.

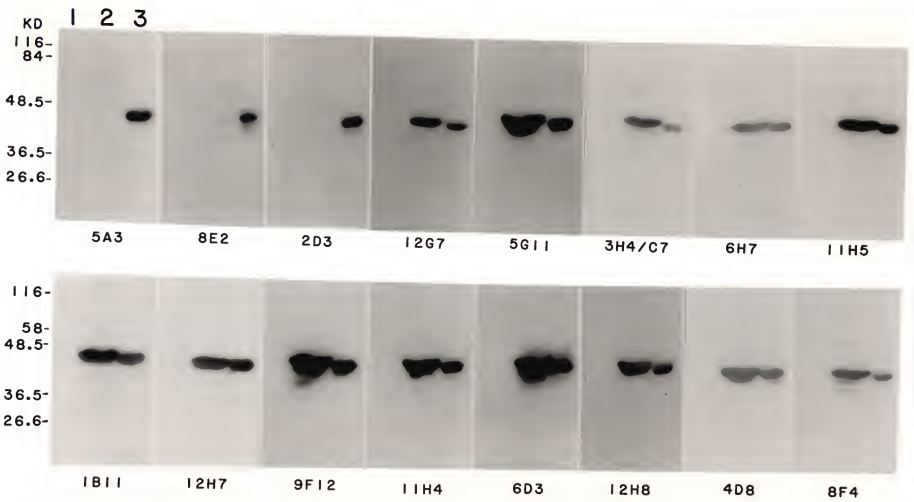
The two bacterial plasmin binding proteins were compared for any evidence of antigenic relatedness using sixteen mouse monoclonal antibodies, prepared against group C streptokinase. These experiments were carried out by Western blot analysis. The results of these experiments are summarized in Figure 5-5. All the monoclonal antibodies tested reacted efficiently with group C streptokinase as expected (see lane 3 of each Western blot). Thirteen of the sixteen monoclonal antibodies reacted equally well with the streptokinase protein from strain 64/14 present in the concentrated culture supernatant see lane 2 of each Western blot. However none of the monoclonal antibodies examined reacted significantly with the extracted 41,000 dalton plasmin receptor, see lane 1 of each Western blot.

These studies demonstrated certain unique epitopes present on group C streptokinase molecules that are not present on the group A plasminogen activator molecule. Overall the group A and group C streptokinase proteins were found to be antigenically closely related, while the group A plasmin receptor was totally devoid of any of the antigenic determinants found on streptokinase.

Discussion

The purpose of this investigation was to compare the streptococcal strain 64/14 receptor for human plasmin with the well characterized secreted plasmin(ogen) binding streptococcal protein streptokinase, to

Figure 5-5. Analysis of the antigenic relationship of the 64/14 plasmin receptor and 64/14 streptokinase and group C streptokinase with mouse anti-group C streptokinase monoclonal antibodies. Parallel protein samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred to separate nitrocellulose membranes. Each individual blot was probed individually in a sandwich assay first with an anti-group C streptokinase monoclonal antibody, followed by goat, anti-mouse IgG antibody, followed by ^{125}I-Protein G. The resulting blots were autoradiographed at -70°C for 6 to 8 hours with intensifying screens. The identity of each monoclonal antibody is depicted below each blot. Lane 1 of each blot contains extracted plasmin receptor protein (approx. 5.0 μg). Lane 2 of each blot contains 64/14 concentrated culture supernatant containing approx. 2.0 μg of streptokinase. Lane 3 of each blot contains 2.0 μg of group C streptokinase (Kabikinase). See Methods for precise experimental details.



Lane 1. Plasmin Receptor Protein

Lane 2. Group A SK

Lane 3. Group C SK

explore any possible similarities or differences. Streptokinase is a unique plasminogen activator. Unlike eukaryotic plasminogen activators, streptokinase has no enzymatic activity. The activation mechanism lies in its ability to form a specific 1:1 stoichiometric complex with plasminogen, as well as with plasmin, which leads to the formation of an active enzyme moiety presumably through conformational changes in the plasminogen molecule without the cleavage event at the Arg_{560}-Val_{561} peptide bond (Markus et al., 1976), that can in turn act as a plasminogen activator for other plasminogen molecules. This is a function neither of the two proteins alone possesses.

The data presented in Chapters Two, Three, and Four provides good evidence that the surface associated plasmin binding receptor activity is distinct from the streptococcal plasminogen activator (streptokinase). While both the plasmin receptor and streptokinase display a high affinity interaction towards plasmin; the plasmin receptor specifically recognizes plasmin while demonstrating no significant reactivity with the native zymogen precursor Glu-plasminogen (see Chapters Two and Three). By contrast streptokinase displays similar affinity for plasminogen and plasmin.

This investigation focused on obtaining plasmin receptor molecules and secreted streptokinase from a single streptococcal strain 64/14 culture, and comparing the two molecules by three criteria: (1) functionally, by measuring plasminogen activator activity; (2) plasmin binding specificity using (a) ^{125}I-plasmin with and without EACA, (b) plasmin heavy chain and (c) plasmin light chain as probes; and (3) antigenically, by the production of a polyclonal anti-plasmin receptor antibodies as well as testing a series of mouse anti-streptokinase

monoclonal antibodies. All of these studies were carried out using a Western blotting approach to enable specific molecular species to be identified and compared in each of these assays. In addition to using the group A strain 64/14 as a source of streptokinase, five other sources (three group A and 2 group C) were included for comparison. In all studies, the commercially available, highly purified group C streptokinase (Kabikinase) was included as a reference.

In agreement with my earlier work, the extracted plasmin receptor preparation did not possess plasminogen activator activity (Table 5-1). This lack of plasminogen-activator potential was further demonstrated using a solid-phase plasminogen-activator assay (see Panel A of Figure 5-1) in which plasminogen activator molecules could be identified and correlated to a molecular size. The assay identified the streptokinase produced by strain 64/14, having an M_r of 48,000 daltons which comigrated with Kabikinase and 3 of the other streptokinases examined. The group A strain B923 secreted a slightly smaller streptokinase molecule (see Panel A of Figure 5-1). When these preparations were Western blotted and probed with ^{125}I-plasmin the 41,000 dalton plasmin binding activity could be clearly identified (see Panel B of Figure 5-1) and shown to exhibit the sensitivity to EACA previously reported (see Panel C of Figure 5-1). However, the streptokinase proteins including Kabikinase, and the strain 64/14 streptokinase were not inhibited from binding ^{125}I-plasmin in the presence of EACA (Compare Panels B and C of Figure 5-1).

The results of probing these Western-blotted proteins with either ^{125}I-plasmin heavy chain or ^{125}I-plasmin light chain were not as conclusive. Previous data (see Chapter Three) suggested that the 41,000 dalton protein would be detected preferentially by the heavy chain

probe. However, it appeared that either heavy or light chain would detect the 41,000 dalton protein by Western blot assay. In agreement with work by Summaria and Robbins (1976) the group C streptokinase (Kabikinase), as well as the streptokinase produced by strain ATCC 12449 were preferentially detected by probing Western blots with ^{125}I-light chain (see Panel B of Figure 5-2). Surprisingly, not all the streptokinases were seen to react preferentially with ^{125}I-light chain.

The comparison of streptokinase and plasmin receptor indicated no antigenic relatedness. Polyclonal rabbit anti-streptokinase antibody and both polyclonal rabbit and polyclonal mouse anti-plasmin receptor antibody were prepared. These antibodies were tested for reactivity to the extracted plasmin receptor and several streptokinases. The rabbit anti-plasmin receptor antibodies showed no cross-reactivity with streptokinase from the strain 64/14, or with two other group A streptokinases, or any of the three group C streptokinase sources studied (Figure 5-3). Two polyclonal mouse anti-plasmin receptor antibody preparations also showed no cross-reactivity with 64/14 streptokinase or with the prototype group C streptokinase (Kabikinase)(Figure 5-4). The polyclonal rabbit anti-group C streptokinase antibody detected all forms of streptokinases examined, while showing no cross-reactivity with the 41,000 dalton plasmin binding protein in the extract of strain 64/14.

Furthermore, 16 mouse monoclonal antibodies were tested for reactivity towards the strain 64/14 streptokinase, the extracted plasmin receptor, and group C streptokinase (Kabikinase). Thirteen of the sixteen monoclonal antibodies recognized the 48,000 dalton 64/14 streptokinase. As expected, all the monoclonal antibodies reacted with

group C streptokinase (Kabikinase), however, none could detect the 41,000 dalton plasmin binding protein in the extract of strain 64/14 (Figure 5-5).

Taken together these findings provide evidence that the single group A streptococcal strain 64/14 produces two proteins with affinity for human plasmin(ogen), that can be shown to be physicochemically, functionally, and antigenically distinct.

CHAPTER SIX
SUMMARY AND CONCLUSIONS

This dissertation describes the first report of a prokaryotic cellular receptor for the human fibrinolytic enzyme plasmin. This study was designed to characterize the plasmin receptor more completely in order to explore what role, if any, it might play in streptococcal infections. The specific goals of the study were to: (1) identify and characterize a group A streptococcal receptor for human plasmin; (2) map the binding site on human plasmin recognized by the bacterial plasmin receptor; (3) isolate and purify a functionally active group A streptococcal plasmin receptor; and (4) compare the group A streptococcal plasmin receptor to the known streptococcal plasmin(ogen) binding protein streptokinase.

Plasmin is the key component of the mammalian fibrinolytic enzyme system which is responsible for intravascular blood clot lysis. The two chain (heavy and light) plasmin serine protease, which cleaves fibrin, is derived from the circulating single chain zymogen precursor Glu-plasminogen. This derivation is brought about by plasminogen activators, which cleave a single peptide bond in the plasminogen molecule (see Chapter One for a detailed discussion of plasminogen activation). The light chain of the plasmin molecule contains the enzyme active site (Robbins and Summaria, 1970; Wiman, 1977). The plasmin heavy chain contains five homologous triple loop structures known as kringles, which are responsible for fibrin binding (Sottrup-Jensen et al., 1978).

Streptococci and streptococcal products have been known to interact with the fibrinolytic system for many years (Tillett and Sherry, 1949). The secreted streptococcal plasminogen activator, streptokinase, was identified by Tillett and Garner in 1933. This protein is known to bind to both human plasminogen and plasmin with high affinity (Reddy and Markus, 1972), through interactions with the light chain of the plasmin molecule (Summaria and Robbins, 1976). The formation of this streptokinase-plasmin(ogen) complex generates an enzymatic moiety capable of plasminogen activator activity, a property neither protein alone possesses.

In the studies documented in Chapter Two, I have identified and characterized a group A streptococcal surface receptor that binds human plasmin while demonstrating no significant affinity for the zymogen form of the molecule Glu-plasminogen. The expression of this binding activity was seen with bacteria grown in either Todd-Hewitt broth or chemically defined media. The binding of plasmin to its bacterial receptor did not inhibit its enzymatic activity. In fact, bacterial bound plasmin was shown to be capable of cleaving both synthetic substrates, as well as its natural substrate, fibrin. The bacterial bound plasmin was also shown to be fully accessible to small protease inhibitors; specifically, aprotinin, PPACK, and pNpGB. Together, these data suggested that the bound plasmin molecule interacts with the surface of the bacteria in a fashion which leaves the active site accessible to substrates. Of great interest was the observation that the bacterial bound plasmin enzyme was not capable of being inhibited by plasmin's main physiological inhibitor α_2-antiplasmin which may have important implications in this bacteria's pathogenic mechanisms.

The plasmin(ogen) molecule contains several characteristic lysine-binding sites (Alkjaersig, 1964; Abiko et al., 1969; Brockway and Castellino, 1972), one high affinity site and four to five sites of lower affinity (Markus et al., 1978a; Markus et al., 1978b). The high affinity site is located in the kringle 1 structure (see Figure 1-1), and one of the lower affinity sites located in the kringle 4 region (Learch et al., 1980). These lysine-binding sites participate in plasmin(ogen) interactions with fibrin (Wiman et al., 1979; Swenson and Thorsen, 1981) and to α_2-antiplasmin (Wiman, 1981). Experiments documented in Chapter Two show that the plasmin-bacterial receptor interaction is one of very high affinity. Also, the plasmin-bacterial receptor interaction, like the first step in the plasmin-α_2-antiplasmin interaction and the plasmin(ogen)-fibrin interaction, was reversibly inhibitable by L-lysine and EACA. Furthermore, plasmin binding to bacterial plasmin receptor was inhibited by α_2-antiplasmin. Together these data suggest the possible involvement of the high affinity lysine binding site in the plasmin-bacterial receptor interaction.

The experiments presented in Chapter Three were designed to examine the nature of the interaction of plasmin with the bacterial plasmin receptor which would account for the particular observations described above. Specific binding to the group A streptococcus, 64/14, was demonstrated with plasmin's heavy chain. However, the isolated heavy chain alone was not as efficient a competitor as intact Lys-plasmin. It was noted that although heavy chain alone could completely inhibit Lys-plasmin binding to bacterial plasmin receptor, in competition assays, none of the isolated lysine-binding kringle domains alone or in combination had any significant inhibitory effect. These data stressed

the importance of the conformation of the entire heavy chain for optimal plasmin binding to bacterial receptor. Therefore the bacterial binding of plasmin differs from the kind of interactions seen with α_2-antiplasmin, fibrin and fibrinogen. Supporting the hypothesis that a particular conformation was a requirement for optimal binding were the data, also presented in Chapter Three, that demonstrated the conformationally distinct form of the native Glu-plasminogen zymogen (Lys-plasminogen) could specifically interact with the bacterial plasmin receptor without converting to Lys-plasmin.

Taken together these observations indicate that the binding of plasmin to the bacterial plasmin receptor is dependent on the conformation of the plasmin molecule, and involves interactions that are distinct from those occurring between other known plasmin(ogen) binding molecules like fibrin, fibrinogen, α_2-antiplasmin, thrombospondin, histidine-rich glycoprotein, and streptokinase.

The plasmin receptor activity expressed on the surface of the group A streptococcal strain 64/14 bacteria was isolated and purified to functional homogeneity to further characterize and distinguish it as a unique molecule. The isolation and purification results are the subject of Chapter Four.

Of a variety of different extraction techniques employed, treatment of the bacteria with the enzyme mutanolysin yielded the highest quantity of soluble plasmin binding activity. This soluble plasmin binding activity was predominantly associated with a 41,000 dalton molecule identified by Western blot analysis using ^{125}I-plasmin as a probe. In agreement with the earlier data, the soluble plasmin binding activity was inhibited by L-lysine and EACA, in both dot-blot and Western blot assays

with ^{125}I-plasmin. The 41,000 dalton molecule was specifically purified from the mutanolysin extract of strain 64/14 by affinity chromatography with immobilized plasmin and elution with L-lysine or EACA. Analysis of the affinity purified plasmin binding activity by SDS-PAGE and Western blotting, demonstrated that 41,000 dalton molecule was the predominant molecule recovered, as detected by silver staining, and this molecule was also responsible for the ^{125}I-plasmin binding activity of the affinity purified sample. This plasmin binding molecule could be destroyed by trypsin digestion and was consequently protein in nature. The 41,000 dalton plasmin binding protein was demonstrated to be totally devoid of plasminogen activator activity, distinguishing it from the secreted plasmin(ogen) binding protein streptokinase.

A detailed comparison between the plasmin receptor activity and streptokinase produced by the same group A strain are the focus of the studies in Chapter Five. The purpose of this series of experiments was to make a comparison of the streptococcal strain 64/14 plasmin receptor with the known and well characterized secreted streptococcal plasmin(ogen) binding protein streptokinase. These studies focused on obtaining plasmin receptor molecules as well as secreted streptokinase from a single culture of streptococcal strain 64/14. These two molecules were then compared for: (1) functional activity, (2) plasmin binding specificity, and (3) antigenic relatedness These studies were performed using Western blot assays to enable specific molecular species to be identified and compared directly to function or antigenic reactivity. In addition to streptokinase from strain 64/14, five other sources of streptokinase were included in these studies for a more complete comparison.

In agreement with the earlier data, the extracted plasmin receptor preparation did not possess plasminogen activator activity. The streptokinase proteins from all strains examined, including strain 64/14, were identified using a solid-phase plasminogen activator assay. This assay also allowed the molecular weight of the plasminogen activator to be defined. The 41,000 dalton plasmin receptor lacked the ability to activate plasminogen. A further distinction between the streptokinase proteins and the plasmin receptor was observed when the proteins were probed with ^{125}I-plasmin in the presence of EACA. All of the streptokinase proteins examined bound ^{125}I-plasmin equally well in the presence of EACA while the bacterial plasmin receptor failed to bind plasmin in the presence of EACA.

The antigenic relatedness of the plasmin receptor and streptokinase was compared using polyclonal antibodies prepared in rabbits and mice to the purified 41,000 dalton plasmin receptor. Mouse and rabbit polyclonal antibodies were prepared that could recognize the 41,000 dalton protein while showing no reactivity with the 48,000 dalton plasminogen activator molecule (streptokinase) produced by the same group A isolate from which the plasmin receptor activity had been isolated. The 48,000 dalton, strain 64/14, streptokinase was found to be reactive with a rabbit polyclonal antibody to group C streptokinase. This antibody recognized the plasminogen activator molecule (streptokinase) produced by three group A and two group C isolates that were studied. This antibody failed to recognize any epitopes on the 41,000 dalton plasmin receptor molecule. Furthermore none of 16 monoclonal antibodies prepared to different epitopes of a group C streptokinase reacted with the plasmin receptor. Thirteen of these antibodies recognized epitopes on the strain 64/14

group A streptokinase while three antibodies recognized epitopes on group C streptokinase which were not present on the group A plasminogen activator molecule. These findings further confirm the antigenic difference noted previously between streptokinases of group A and group C streptococci (Dillon and Wannamaker, 1965; Huang et al., 1989). These studies demonstrate that not only does the group A strain 64/14 produce two distinct proteins with high affinity for human plasmin but there is no evidence that these proteins share any common structural features.

This dissertation describes the first report of a prokaryotic cellular receptor for human plasmin. However, eukaryotic cellular receptors for plasmin(ogen) have been reported recently (Hajjar et al., 1986; Miles et al., 1986; Miles and Plow 1985; Plow et al., 1986). Eukaryotic cellular receptors for the plasminogen activators urokinase-type plasminogen activator (Bajpai and Baker, 1985; Del Rosso et al., 1985; and Vassalli et al., 1985) and tissue-type plasminogen activator (Beebe, 1987; and Hajjar et al., 1987) have also been described. It has been concluded by others that the expression of plasminogen receptors and their colocalization with urokinase- and tissue-type plasminogen activator receptors provide cells with a basic mechanism for obtaining proteolytic activity for the purposes of certain cellular functions (Knox et al., 1987) and for modification of their surroundings (Knudsen et al., 1986; Sheela and Barret, 1982). It had long been recognized (Carrel and Burrows, 1911; Lambert and Hanes, 1911) that cancer tissue consistently caused proteolytic degradation upon culturing. It has since been shown that this proteolytic activity stems from the ability of many types of cancer cells to produce and secrete plasminogen activators. In fact plasmin, in addition to acting within the fibrinolytic system, has

been demonstrated to activate complement components, hydrolyze components of connective tissue and basement membranes such as laminin (Liotta et al., 198la; Ott et al., 1981), fibronectin (Liotta et al., 1981), proteoglycans (Emonds-Alt et al., 1980), thrombospondin (Lawler and Slayter, 1981) and type-V collagen (Liotta et al., 198la,b), as well as proteolytically activating other latent proteases like collageneases (O'Grady et al., 1981; Stricklin et al., 1977; and Werb et al., 1977). These collagenases can then catalyze the initial cleavage of the collagen molecules, which are then susceptible to further proteolytic action by plasmin (Danø, 1985). It is now recognized that many normal cell types also have the ability to produce plasminogen activators, as well as the ability to specifically bind the plasminogen activators and plasmin(ogen).

In conclusion, the ability of certain streptococcal bacteria, like strain 64/14, to both produce a plasminogen activator (i.e., streptokinase) and also to express a receptor having an extremely high affinity for the activation product plasmin may be important for certain of the invasive properties of these organism. Furthermore, since plasmin bound to a group A streptococcus is incapable of inhibition by plasmin's normal physiological regulator α_2-antiplasmin, the bacterium has acquired a non-regulatable proteolytic activity that may contribute to its tissue-invasive properties. All of these findings would be consistent with a linked role for streptokinase and the surface bacterial plasmin receptor. Together these properties of a group A streptococcus would provide the bacteria with a mechanism to capture an unregulated enzyme activity that might alter their ability to interact with host barriers. In fact, the streptococcal strain 64/14 when grown in human

plasma has been documented to both be capable of generating plasmin from plasminogen, as well as binding the active enzyme to its surface (DesJardin et al., 1989). The importance of this selective receptor to the infectious disease process of receptor positive bacteria remains to be established.

REFERENCES

Abiko, Y., M. Iwamoto, and M. Tomikawa. 1969. Pasminogen-plasmin system V: A stoichiometric equilibrium complex of plasminogen and a synthetic inhibitor. Biochim. Biophys. Acta. 185:424-431.

Alkjaersig, N. 1964. The purification and properties of human plasminogen. Biochem. J. 93:171-182.

Aoki, N., M. Moroi, M. Matsuda, and K. Tachiya. 1977. The behavior of α_2-plasmin inhibitor in fibrinolytic states. J. Clin. Invest. 60:361-369.

Astrup, T. 1978. Fibrinolysis: An overview. In: Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, pp. 1-57, Davidson, J.F., Towan, R.M., Samana, M.M. and Desnoyers, P.C. (eds.), Raven Press, New York.

Atichartakarn, V., V.J. Marder, E.P. Kirby, and A.Z. Budzynski. 1978. Effects of enzymatic degradation on the subunit composition and biologic properties of human factor VIII. Blood 51:281-297.

Bajpai, A., and J.B. Baker. 1985. Cryptic urokinase binding sites on human foreskin fibroblasts. Biochem. Biophys. Res. Commun. 133:475-482.

Beebe, D.P. 1987. Binding of tissue plasminogen activator to human umbilical vein endothelial cells. Thromb. Res. 46:241-254.

Beers, W.H., S. Strickland, and E. Reich. 1975. Ovarian plasminogen activator: Relationship to ovarian and hormonal regulation. Cell 6:387394.

Bhaduri, S., and Demchick, P.H. 1983. Simple and rapid method for disruption of bacteria for protein studies. Applied and Environ. Microbiol. 46:941-943.

Boyle, M.D.P., and K.J. Reis. 1987. Bacterial Fc receptors. Biotechnology 5:697-703.

Brockway, W., and F. Castellino. 1972. Measurement of the binding of antifibrinolytic amino acids to various plasminogens. Arch. Biochem. Biophys. 151:194-199.

Broeseker, T.A., M.D.P. Boyle, and R. Lottenberg. 1988. Characterization of the interaction of human plasmin with its specific receptor on a group A streptococcus. Microbial Patho. 5:19-27.

Brogden, R.N., T.M. Speight, and G.S. Avery. 1973. Streptokinase: A review of its clinical pharmacology, mechanism of action and therapeutic uses. Drugs 5:357-445.

Burova, L., P. Christensen, R. Grubb, A. Johnson, G. Samuelsson, C. Schalen, and M.L. Svenson. 1980. Changes in virulence, M protein and IgG Fc receptor activity in a type 12 group A streptococcal strain during mouse passage. Acta Path. Microbiol. Scand. 88:199-205.

Burova, L., P. Christensen, R. Grubb, I.S. Krasilnikov, G. Samuelsson, C. Schalen, M.L. Svenson, and U. Zatterstrom. 1981. IgG-Fc receptors in T-type 12 group A streptococci from clinical specimens: Absence from M-type 12 and presence in M-type 22. Acta Path. Microbiol. Scand. 89:433-435.

Carrel, A., and M.T. Burrows. 1911. Cultivation in vitro of malignant tumors. J. Exp. Med. 13:571-575.

Castellino, F.J., Sodetz, J.M., Brockway, W.J., and Sefring, G.E., Jr. 1976. Streptokinase. Methods Enzymol. 45:244-257.

Cederholm-Williams, S.A. 1977. The binding of plasminogen (mol. wt. 84,000) and plasmin to fibrin. Thromb. Res. 11:421-423.

Cederholm-Williams, S.A., F. DeCock, H.R. Lijnen, and D. Collen. 1979. Kinetics of the reactions between streptokinase, plasmin and α_2-antiplasmin. Eur. J. Biochem. 100:125-132.

Chiles, T.C., T.W. O'Brien, and M.S. Kilberg. 1987. Production of monospecific antibodies to a low-abundance hepatic membrane protein using nitrocellulose immobilized protein as antigen. Anal. Biochem. 163:136-142.

Christensen, L.R. 1945. Streptococcal fibrinolysis: Proteolytic reaction due to serum enzyme activated by streptoccus fibrinolysin. J. Gen. Physiol. 28:363-383.

Christensen, L.R., and C.M. Macleod. 1945. Proteolytic enzyme of serum: Characterization, activation and reaction with inhibitors. J. Gen. Physiol. 28:559-583.

Christensen, U., and I. Clemmenson. 1977. Kinetic properties of the primary inhibitor of plasmin from human plasma. Biochem. J. 163:389-391.

Cleary, P., S. Jones, J. Robbins, and W. Simpson. 1987. Phase variation and M-protein expression in group A streptococci. In: Streptococcal Genetics, p.102, Ferretti, J.J., and R. Curtiss III (eds.), American Society for Microbiology, Washington, D.C..

Cleland, W.W. 1967. The statistical analysis of enzyme kinetic data. Adv. Enzymol. 29:1-32.

Collen, D. 1976. Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. Eur. J. Biochem. 69:209-216.

Collen, D. 1980. On the regulation and control of fibrinolysis. Thromb. and Hemostat. 43:77-89.

Collen, D., F. DeCock, and M. Verstralte. 1975. Immunochemical distinction between antiplasmin and α_1-antitrypsin. Thromb. Res. 7:245-249.

Danø, K., P.A. Andreasen, J. Grondahl-Hansen, P. Kristensen, L.S. Neilsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. Adv. Cancer Res. 44:139-267.

Davies, M.C., M.E. Englert, and E.C. DeRenzo. 1964. Interaction of streptokinase and human plasminogen. J. Biol. Chem. 239:2651-2656.

Del Rosso, M., G. Dini, and G. Fibbi. 1985. Receptors for plasminogen activator, urokinase, in normal and Rous Sarcoma virus-transformed mouse fibroblasts. Cancer Res. 45:630-636.

DesJardin, L., M.D.P. Boyle, and R. Lottenberg. 1988. Group A streptococci grown in human plasma generate and bind plasmin to a specific surface receptor. In: Proceedings of the 88th Annual Meeting of the American Society for Microbiology, p. 57, B-169a, Hartman, P.A., and Morello, J.A. (eds), American Society for Microbiology, Washington, D.C..

Diano, M., A. Le Bivic, and M. Hirn. 1987. A method for the production of highly specific polyclonal antibodies. Anal. Biochem. 166:224229.

Dillon, H.C., and L.W. Wannamaker. 1965. Physical and immunological differences among streptokinases. J. Exp. Med. 121:351-371.

Edy, J., and D. Collen. 1979. The Interaction in human plasma of antiplasmin, the fast-reacting plasmin inhibitor with plasmin, thrombin, trypsin, and chymotrypsin. Biochim. Biophys. Acta 484:423-432.

Elliott, S.D., and V.P. Dole. 1947. An inactive precursor of streptococcal proteinase. J. Exp. Med. 85:305-310.

Emonds-Alt, X., E. Quisquater, and G. Vaes. 1980. Proteoglycan- and fibrin-degrading neutral proteinase activities of Lewis lung carcinoma cells. Eur. J. Cancer 16:1257-1261.

Fritz, H., and G. Wunderer. 1983. Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneim.-Forsch./Drug Res. 33:479-494.

Ginsburg, I. 1972. Mechanisms of cell and tissue injury induced by group A streptococci: Relation to post-streptococcal sequelae. J. Infect. Dis. 126:419-456.

Groskopf, W.R., L. Summaria, and K.C. Robbins. 1969. Studies on the active center of human plasmin: Partial amino acid sequence of a peptide containing the active center serine residue. J. Biol. Chem. 244:3590-3597.

Hajjar, K.A., N.M. Hamel, P.C. Harpel, and R.L. Nachman. 1987. Binding of tissue plasminogen activator to cultured human endothelial cells. J. Clin. Invest. 80:1712-1719.

Hajjar, K.A., P.C. Harpel, E.A. Jaffe, and R.L. Nachman. 1987. Binding of plasminogen to cultured human endothelial cells. J. Biol. Chem. 261:11656-11662.

Haupt, H., and Heimburger, N. 1972. Humanserumproteine mit hoher affinitat zu carboxymethylcellulose I. Hoppe-Seyler's Z. Physiol. Chem. 353:1125-1132.

Hayes, M.L., and F.J. Castellino. 1979a. Carbohydrate of human plasminogen variants-I. Carbohydrate composition, glycopeptide isolation and characterization. J. Biol. Chem. 254:8768-8771.

Hayes, M.L., and F.J. Castellino. 1979b. Carbohydrate of human plasminogen variants-II. Structure of the asparagine-linked oligosaccharide unit. J. Biol. Chem. 254:8772-8776.

Hayes, M.L., and F.J. Castellino. 1979c. Carbohydrate of the human plasminogen variants-III. Structure of the O-glucosidically linked oligosaccharide unit. J. Biol. Chem. 254:8777-8780.

Holvoet, P., H.R. Lijnen, and D. Collen. 1985. A monoclonal antibody specific for lys-plasminogen. J. Biol. Chem. 260:12106-12111.

Huang, T-T., H. Malke, and J.J. Ferretti. 1989. Heterogeneity of the streptokinase gene in group A streptococci. Infec. Immun. 57:502-506.

Hunter, R.L., and B. Bennett. 1984. The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and octablock copolymers. J. Immunol. 133:3167-3175.

Johnston, K.H., and J.B. Zabriskie. 1986. Purification and partial characterization of the nephritis strain-associated protein from Streptococcus pyogenes group A. J. Exp. Med. 163:697-711.

Johnston, R.B. 1984. Current concepts: recurrent bacterial infection in children. N. Engl. J. Med. 310:1237-1243.

Jones, P.A., and Y.A. DeClerck. 1980. Destruction of extracellular matrices containing glycoproteins, elastin, and collagen by metastatic human tumor cells. Cancer Res. 40:3222-3227.

Kaplan, M.H. 1944. Nature and role of the lytic factor in hemolytic streptococcal fibrinolysis. Proc. Soc. Exp. Biol. and Med. 57:40-43.

Kay, A.B., D.S. Petter, and R. McKenzie. 1974. The identification of fibrinopeptide B as a chemotactic agent derived from human fibrinogen. Brit. J. Haematol. 27:669-677.

Kettner, C., C. Mirabelli, J.V. Pierce, and E. Shaw. 1980. Active site mapping of human and rat urinary kallikreins by peptidyl chloromethyl ketones. Arch. Biochem. Biophys. 202:420-430.

Knox, P., S. Crooks, M.C. Scaife, and S. Patel. 1987. Role of plasminogen, plasmin, and plasminogen activators in the migration of fibroblasts into plasma clots. J. Cell. Physiol. 132:501-508.

Knudsen, B.S., R.L. Silverstein, L.L.K. Leung, P.C. Harpel, and R.L. Nachman. 1986. Binding of plasminogen to extracellular Matrix. J. Biol. Chem. 261:10765-10771.

Kronvall, G. 1973. A surface component in group A, C, and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. 111:1401-1406.

Kronvall, G., C. Schonbeck, and E. Myhre. 1979. Fibrinogen binding structures in B-hemolytic streptococci group A,C, and G. Acta. Path. Microbiol. Scand., Sect. B, 87:303-310.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680-685.

Lambert, R.A., and F.M. Hanes. 1911. Characteristics of growth of sarcoma and carcinoma cultivated in vitro. J. Exp. Med. 13:495-504.

Lancefield, R.C. 1928. The antigenic complex of Streptococcus haemolyticus. I. Demonstration of a type specific substance in extracts of Streptococcus haemolyticus. J. Exp. Med. 47:91-103.

Laurell, C.B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15:45-52.

Lawler, J., and H. Slayter. 1981. The release of heparin binding peptides from platelet thrombospondin by proteolytic action of thrombin, plasmin and trypsin. Thromb. Res. 22:267-279.

Lerch, P.G., and E.G. Rickli. 1980. Studies on the chemical nature of lysine-binding sites and their localization in human plasminogen. Biochim. Biophys. Acta 625:374-378.

Lerch, P.G., E.G. Rickli, W. Lergier, and D. Gillessen. 1980. Localization of individual lysine-binding regions in human plasminogen and investigations on their complex-forming properties. Eur. J. Biochem. 107:7-13.

Leung, L.L.K., R.L. Nachman, and P.C. Harpel. 1984. Complex formation of platelet thrombospondin with histidine-rich glycoprotein. J. Clin. Invest. 73:5-12.

Levy, H.B., and Sober, H.A. 1960. A simple chromatographic method for preparation of gamma globulin. Proc. Soc. Exp. Bio. and Med. 103:250-252.

Lijnen, H.R., Hoylaerts, M., and Collen, D. 1980. Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. J. Biol. Chem. 255:10244-10222.

Liotta, L.A., R.H. Goldfarb, R. Brundage, G.P. Siegal, V. Terranova, and S. Garbisa. 1981a. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous of basement membrane. Cancer Res. 41:4629-4636.

Liotta, L.A., R.H. Goldfarb, and V.P. Terranova. 1981b. Cleavage of laminin by thrombin and plasmin: Alpha thrombin selectively cleaves the beta chain of laminin. Thromb. Res. 21:663-673.

Liu, T-Y., and S.D. Elliott. 1965. Streptococcal proteinase: The zymogen to enzyme transformation. J. Biol. Chem. 240:1138-1144.

Lottenberg, R., C.C. Broder, and M.D.P. Boyle. 1987. Identification of a specific receptor for plasmin on a group A streptococcus. Infect. Immun. 55:1914-1918.

Lottenberg, R., F.R. Dolly, and C.S. Kitchens. 1985. Recurring thromboembolic disease and pulmonary hypertension associated with severe hypoplasminogenemia. Am. J. Hematol. 19:181-193.

Malinowski, D.P., J.E. Sadler, and E.W. Davie. 1984. Characterization of a complementary deoxyribonucleic acid coding for human and bovine plasminogen. Biochemistry 23:4243-4250.

Marder, V.J., S.E. Martin, and R.W. Calman. 1982. Clinical aspects of consumptive thrombohemorrhagic disorders. In: Hemostasis and Thrombosis: Basic Principles and Clinical Practice, p. 672, R.W. Colman, J. Hirsh, V.J. Mardar and E.W. Solzman (eds.), J.B. Lippincott Co., Philadelphia.

Markus, G., J.L. DePosquale, and F.C. Wissler. 1978a. Quantitative determination of the binding of ϵ-aminocaproic acid to native plasminogen. J. Biol. Chem. 253:727-732.

Markus, G., J.L. Evers, and G.H. Hubika. 1976. Activator activities of the transient forms of the human plasminogen streptokinase complex during its proteolytic conversion to the stable activation complex. J. Biol. Chem. 251:6495-6504.

Markus, G., J.L. Evers, and G.H. Hubika. 1978b. Comparison of some properties of native (Glu) and modified (Lys) human plasminogen. J. Biol. Chem. 253:733-739.

Markwell, M.A.K. 1982. A new solid-state reagent to iodinate proteins. Anal. Biochem. 125:427-432.

McClintock, D.K., and P.H. Bell. 1971. The mechanism of activation of human plasminogen by streptokinase. Biochem. Biophys. Res. Commun. 3:694-702.

Merril, C.R., D. Goldman, S.A. Sedman, and M.H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211:1437-1438.

Miles, L.A., E.G. Levin, and E.F. Plow. 1986. Localization of fibrinolytic proteins on peripheral blood cells and endothelial cells. Circulation 74: Suppl. II:246a.

Miles, L.A., and E.F. Plow. 1985. Binding and activation of plasminogen on the platelet surface. J. Biol. Chem. 260:4303-4311.

Miles, L.A., and E.F. Plow. 1987. Receptor mediated binding of the fibrinolytic components, plasminogen and urokinase to peripheral blood cells. Thromb. Haemost. 58:936-942.

Milstone, H. 1941. A factor in normal human blood which participates in streptococcal fibrinolysis. J. Immunol. 42:109-116.

Mori, M., and N. Aoki. 1976. Isolation and characterization of α_2-plasmin inhibitor from human plasma. J. Biol. Chem. 251:5956-5965.

Myhre, E.B., and P. Krusela. 1983. Binding of human fibronectin to group A,C, and G streptococci. Infect. Immun. 40:29-34.

Nilsson, T., I. Sjöholm, and B. Wiman. 1982. Circular dichroism studies on α_2-antiplasmin and its interaction with plasmin and plasminogen. Biochem. Biophys. Acta. 705:264-270.

O'Grady, R.L., L.I. Upfold, and R.W. Stephens. 1981. Rat mammary carcinoma cells secrete active collagenase and active latent enzyme in the stroma via plasminogen activator. Int. J. Cancer 28:509-515.

Ohkuni, H., J. Friedman, I. Van De Rijn, V.A. Fischetti, T. Poon-King, and J.B. Zabriskie. 1983. Immunological studies of post-streptococcal sequelae: Serological studies with an extracellular protein associated with nephritogenic streptococci. Clin. Exp. Immunol. 54:185-193.

Ohlsson, K., and D., Collen. 1977. Comparison of the reactions of neutral granulocytic proteases with the major plasma protease inhibitors and with antiplasmin. Scad. J. Clin. Lab. Invest. 37:345-350.

Ott, U., E. Odermatt, J. Engel, H. Furthmayr, and R. Timpl. 1982. Protease resistance and conformation of laminin. Eur. J. Biochem. 123:63-72.

Plow, E.F., D.E. Freaney, J. Plescia, and L.A. Miles. 1986. The plasminogen system and cell surfaces: Evidence for plasminogen and urokinase receptors on the same cell type. J. Cell. Biol. 103:2411-2420.

Rakoczi, I., B. Wiman, and D. Collen. 1978. On the biological significance of the specific interaction between fibrin, plasminogen and antiplasmin. Biochem. Biophys. Acta. 540:295-300.

Reddy, K.N.N., and G. Markus. 1972. Mechanism of activation of human plasminogen by streptokinase. J. Biol. Chem. 247:1683-1691.

Reis, K.J., E.M. Ayoub, and M.D.P. Boyle. 1983. Detection of receptors for the Fc region of IgG on streptococci. J. Immunol Methods. 59:83-94.

Reis, K.J., E.M. Ayoub, and M.D.P. Boyle. 1984. Streptococcal Fc receptors: I. Isolation and partial characterization of the receptor from a group C streptococcus. J. Immunol. 132:3091-3098.

Reis, K.J., G.O. Von Mering, M.A. Karis, E.L. Faulmann, R. Lottenberg, and M.D.P. Boyle. 1988. Enzyme-labeled type III bacterial Fc receptors: A versatile tracer for immunoassay. J. Immunol. Meth. 107:273-280.

Reis, K.J., M. Yarnall, E.M. Ayoub, and M.D.P. Boyle. 1984. Effect of mouse passage on Fc receptor expression by a group A streptococci. Scand. J. Immunol. 20:433-439.

Robbins, K.C. 1981. The regulation and control of the blood fibrinolytic system. In: Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 5, pp. 3-13, Davidson, J.F., Nilsson, I. Mare Astedt, B. (eds.), Churchill Livingstone, Edinburgh.

Robbins, K.C., and L. Summaria. 1970. Human plasminogen and plasmin. Methods Enzymol. 19:184-199.

Robbins, K.C., L. Summaria, B. Hsieh, and R.J. Shah. 1967. The peptide chains of human plasmin: Mechanism of activation of human plasminogen to plasmin. J. Biol. Chem. 242:2333-2342.

Russell-Jones, G.J., E.C. Gotschlich, and M.S. Blake. 1984. A surface receptor specific for human IgA on group B streptococci possessing the Ibc protein antigen. J. Exp. Med. 160:1467-1475.

Ryan, T.J. 1987. Photoaffinity labeling of human plasmin and plasminogen. Pharmac. Ther. 34:335-348.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51:660-672.

Scopes, R.K. 1982. Protein Purification: Principles and Practice, Springer-Verlag, New York.

Sheela, S., and J.C. Barrett. 1982. In vitro degradation of radiolabeled, intact basement membrane mediated by cellular plasminogen activator. Carcinogenesis 3:363-369.

Siegal, J.L., S.F. Hurst, E.S. Liberman, S.E. Coleman, and A.S. Bleiweis. 1981. Mutanolysin-induced spheroplasts of Streptococcus mutans are true protoplasts. Infect. Immun. 31:303-815.

Silverstein, R.L., and R.L. Nachman. 1987. Thrombospondin-plasminogen interactions: Modulation of plasmin generation. Sem. Thromb. and Hemo. 13:335-342.

Sottrup-Jensen, L., N. Claeys, M. Zecler, T.E. Peterson, and S. Magnusson. 1978. Isolation of two lysine-binding fragments and one 'mini'-plasminogen (mw 38,000) by elastase catalyzed-specific limited proteolysis. In: Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, pp. 191-209, Davidson, J.R., Rowan, R.M., Samana, M.M. and Desnoyers, P.C. (eds.), Raven Press, New York.

Sparling, P.F. 1983. Bacterial virulence and pathogenesis: An overview. Rev. Infect. Dis. 5:637-646.

Stief, T.W., P. Lenz, and V. Becker. 1987. A simple method for producing degradation products of fibrinogen by an insoluble derivative of plasmin. Thromb. Res. 48:603-609.

Strickland, S., and W.H. Beers. 1976. Studies on the role of plasminogen activator in ovulation. J. Biol. Chem. 251:5694-5702.

Strickland, S., E. Reich, and M.I. Sherman. 1976. Plasminogen activator in early embryogenesis: Enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Stricklin, G.P., E.A. Baner, J.J. Jeffrey, and A.Z. Eisen. 1977. Human skin collagenase: Isolation of precursor and active forms from both fibroblast and organ cultures. Biochem. 16:1607-1615.

Summaria, L., and K.C. Robbins. 1976. Isolation of a human plasmin derived functionally active light (B) chain capable of forming with streptokinase an equimolar light (B) chain-streptokinase complex with plasminogen activator activity. J. Biol. Chem. 251:5810-5813.

Swenson, E., and Thorsen, S. 1981. Secondary-site binding of Glu-plasmin, Lys-plasmin and mini-plasmin to fibrin. Biochem. J. 197:619- 628.

Switalski, L.M., P. Speziale, M. Hook, T. Waldstrom, and R. Timpl. 1984. Binding of Streptococcus pyogenes to laminin. J. Biol. Chem. 259:3734-3738.

Teger-Nilsson, A.C., P. Friberger, and E. Gyzander. 1977. Determination of a new rapid plasmin inhibitor in human blood by means of a plasmin specific tripeptide substrate. Scand. J. Clin. Lab. Invest. 37:403-409.

Thorsen, S. 1975. Differences in the binding to fibrin of native plasminogen and plasminogen modified by proteolytic degradation: Influence of W-aminocarboxylic acids. Biochem. Biophys. Acta. 393:55-65.

Thorsen, S., I. Clemmensen, L. Sottrup-Jensen, and S. Magnusson. 1981. Absorption to fibrin of native fragments of known primary structure from human plasminogen. Biochem. Biophys. Acta. 668:377-387.

Tillett, W.S., and R.L.L. Garner. 1933. The fibrinolytic activity of hemolytic streptococci. J. Exp. Med. 58:485-502.

Tillett, W.S., and S. Sherry. 1949. The effect in patients of streptococcal fibrinolysin (streptokinase) and streptococcal deoxyribonuclease on fibrinous, purulent and sanguinous pleural exudations. J. Clin. Invest. 28:173-190.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Van De Rijn, I., and R.E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. and Immun. 27:444-448.

Vassallii, J-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell. Biol. 100:86-92.

Violand, B.N., J.M. Sodetz, and F.J. Castellino. 1975. The effect of ϵ-Amino Caproic Acid on the gross conformation of plasminogen and plasmin. Arch. Biochem. and Biophys. 170:300-305.

Von Mering, G.O., M.D.P. Boyle, C.C. Broder, and R. Lottenberg. 1988. Isolation of a surface receptor for human plasmin from a pathogenic group A streptococcus. Clin. Res. 36:464a.

Wallén, P., and B. Wiman. 1970. Characterization of human plasminogen: I. On the relationship between different molecular forms of plasminogen demonstrated in plasma and found in purified preparations. Biochim. Biophys. Acta. 221:20-30.

Wallén, P., and B. Wiman. 1972. Characterization of human plasminogen: II. Separation and partial characterization of different molecular forms of human plasminogen. Biochim. Biophys. Acta. 257:122-134.

Walz, D.A., T. Bacon-Saguley, S. Kendra-Franczak, and P. DePoli. 1987. Binding of thrombospodin to immobilized ligands - specific interaction with fibrinogen, plasminogen, histidine-rich glycoprotein, and fibronectin. Sem. Thromb. and Hemo. 13:317-325.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by Dodecyl Sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

Weinstein, L. 1953. Antigenic dissimilarity of streptokinases. Proc. Soc. Exp. Biol. Med. 83:689-691.

Werb, Z., C.L. Mainardi, C.A. Vater, and E.D. Harris. 1977. Endogenous activation of latent collagenase by rheumatoid synovial cells: Evidence for a role of plasminogen activator. N. Engl. J. Med. 296:1017-1023.

Wiman, B. 1973. Primary structure of peptides released during activation of human plasminogen by urokinase. Eur. J. Biochem. 39:1-9.

Wiman, B. 1977. Primary structure of the B-chain of human plasmin. Eur. J. Biochem. 76:129-137.

Wiman, B. 1981. Human α_2-Antiplasmin. Methods Enzymol. 80:395-408.

Wiman, B., L. Bowman, and D. Collen. 1978. On the kinetics of the reaction between human antiplasmin and a low- molecular-weight form of plasmin. Eur. J. Biochem. 87:143-146.

Wiman, B., and D. Collen. 1977. Purification and characterization of human antiplasmin, the fast-acting plasmin inhibitor in plasma. Eur. J. Biochem. 78:19-26.

Wiman, B., and D. Collen. 1978. On the kinetics of the reaction between human antiplasmin and plasmin. Eur. J. Biochem. 84:573-578.

Wiman, B., H.R. Lijnen, and D. Collen. 1979. On the specific interaction between the lysine-binding sites in plasmin and complementary sites in α_2-antiplasmin and in fibrinogen. Biochem. Biophys. Acta. 579:142-154.

Wiman, B., and P. Wallén. 1973. Activation of human plasminogen by an insoluble derivative of urokinase. Eur. J. Biochem. 36:25-31.

Wiman, B., and P. Wallén. 1975. Structural relationship between "glutamic acid[1] and lysine " forms of human plasminogen and their interaction with the NH_2-terminal alteration peptide as studied by affinity chromatography. Eur. J. Biochem. 50:489-494.

Winn, E.S., S.P. Hu, S.M. Hochschwender, and R.A. Laursen. 1980. Studies on the lysine-binding sites of human plasminogen: The effect of ligand structure on the binding of lysine analogs to plasminogen. Eur. J. Biochem. 104:579-586.

Woodard, L.F., and R.L. Jasman. 1985. Stable oil-in-water emulsions: preparation and uses as vaccine vehicles for lipophilic adjuvants. Vaccine 3:137-144.

Yarnall, M., E.M. Ayoub, and M.D.P. Boyle. 1986. Analysis of surface receptor expression on bacteria isolated from patients with endocarditis. J. Gen. Microbiol. 132:2049-2052.

Yarnall, M., and M.D.P. Boyle. 1986a. Isolation and characterization of typeIIa and type IIb Fc receptors from a group A streptococcus. J. Immunol. 24:549-557.

Yarnall, M., and M.D.P. Boyle. 1986b. Isolation and partial characterization of a type II Fc receptor from a group A streptococcus. Mol. Cell. Biochem. 70:57-66.

Yarnall, M., K.J. Reis, E.M. Ayoub, and M.D.P. Boyle. 1984. An immunoblotting technique for the detection of bound and secreted bacterial Fc receptors. J. Microb. Meth. 3:83-93.

Zolton, R.P., and E.T. Mertz. 1972. Studies on plasminogen. X. Isolation of plasminogen by affinity chromatography using Sepharose butesin. Can. J. Biochem. 50:529-537.

BIOGRAPHICAL SKETCH

Christopher Charles Broder was born on June 12, 1961 in White Plains, New York, the heart of New York suburbia, where he lived for seventeen years. He graduated from White Plains High School in 1979, and having been a member of the 'Jacques Cousteau television generation', went south to attend the Florida Institute of Technology, Melbourne, Florida. He studied marine science for most of his undergraduate career, obtained a Dive Master certification and proceeded to explore every available body of water in the state. He traveled back home in the summers, and worked for Peckham Industries in Connecticut, where he helped finance his academic life by working blacktop. He graduated with a B.S. degree in biology, in 1983, along with Colleen M. Guay whom he would later marry. He stayed on at F.I.T. entering graduate studies, and received his M.S. degree in molecular biology in 1985, and hoped he would never have to return to the wheelbarrow and shovel. In the fall of that year he moved to Gainesville, Florida, and entered graduate school in the department of Immunology and Medical Microbiology at the University of Florida, and began his studies in the laboratory of Dr. Michael D.P. Boyle in 1986. He has completed his research, and expects to receive his Ph.D. in May of 1989 from the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1989

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